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The interference of heparin on IEF-PAGE of erythropoietins

Christian Reichel,* Edmund Benetka, Thomas Geisendorfer, Veronika Scheiblhofer and Friedrich Abzieher

Because of the risk of suffering a stroke or heart attack, some athletes and their medical supervisors admitted having used anticoagulants (e.g. acetylsalicylic acid) in combination with doping with recombinant erythropoietins (rhEPO). Heparin is one of the oldest and cheapest anticoagulants. The anticoagulative effect of heparin is a result of the binding of heparin to the plasma protein antithrombin III and the subsequent inactivation of blood clotting factors (e.g. factor IIa, IXa, Xa, XIa, XIIa). Heparin – a polyanion – is known to interact with carrier ampholytes used in IEF-PAGE. Two different types of heparin pharmaceuticals are used for medical purposes: unfractionated heparins (UFH) and low molecular weight heparins (LMWH). Their influence on IEF- and SDS-PAGE was investigated. Only UFH had a profound impact on IEF-PAGE, leading to excessive smearing or complete abolishment of the EPO IEF-profile and shifting of acidic EPO-isoforms in the endogenous region of the gel. No such effect was observable for SDS-PAGE. Remedies include immunoaffinity purification of EPO before IEF-PAGE or the treatment of the urinary retentate with solid urea. A combined usage of IEF- and SDS-PAGE is recommended for confirming the presence of rhEPO in urine and for further analysis of smearing (and therefore suspicious) samples. This two-method approach is already in accordance with the technical document on EPO-analysis (TD2009EPO) of the World Anti-Doping Agency (WADA).

Keywords: heparin; erythropoietin; doping control; isoelectric focusing; SDS-PAGE

Introduction

Heparin is an anticoagulant which is mainly used for preventive purposes (e.g. during long-distance flights) or immediately after severe thromboembolic events (e.g. deep venous thrombosis (DVT), pulmonary embolism). Two types of pharmaceutical heparin-formulations are currently used – unfractionated heparins (UFH) and low molecular weight heparins (LMWH). The former are mainly used, for example, immediately before or immediately after myocardial infarction; the latter – due to a more easily controllable anticoagulative effect and a less frequently required administration – mainly for preventive and self-administration purposes, for example, before surgery or after DVT.[1] LMWH are prepared by controlled enzymatic (heparinase) or chemical (nitrous acid) cleavage of UFH. The anticoagulative effect of UFH is mostly based on the binding of heparin to antithrombin III which then inactivates activated serine proteases (e.g. the clotting factors IIa (Thrombin), IXa, Xa, XIa, XIIa)[2–3] by complex formation. LMWH on the other hand mainly act via inactivating factor Xa. The difference in the mode of action is directly attributable to the structural differences between UFH (M_r *ca* 5000–30 000 Da, average M_r *ca* 12 000–15 000, average chain length *ca* 45–50 monosaccharides) and LMWH (average M_r *ca* 4000–6500, average chain length *ca* 13–22 monosaccharides): while thrombin inhibition via antithrombin III requires a characteristic pentasaccharide sequence (antithrombin III binding domain) of heparin as well as a minimum chain length of 14–16 monosaccharides, factor Xa inhibition only requires the presence of the pentasaccharide domain. Consequently, LMWH – which partly lack the minimum amount of the 14–16 monosaccharides – possess full anti-Xa but only low anti-IIa activity.[4–6] Acetylsalicylic acid (ASA, Aspirin®) also influences blood coagulation, but not via direct intervention in the blood coagulation cascade but via intervention in the thrombocyte-metabolism – thus preventing aggregation of thrombocytes.[7] A study on the effects of heparins (UFH, LMWH) on the detection of doping with recombinant erythropoietins (rhEPO) became necessary since (1) some athletes and their supervising medical doctors admitted having used anticoagulants (e.g. ASA) for preventive purposes during rhEPO therapies ('blood dilution') and heparin is one of the oldest and cheapest anticoagulants;[8] (2) an interference of heparin with carrier ampholyte-based isoelectric focusing on polyacrylamide gels (CA-IEF-PAGE) – the method used for detecting rhEPO-doping – has been described in scientific literature;[9] (3) more than 100 heparin-binding proteins are known[10–11] – among them cytokines and growth factors (like interleukin 3 (IL-3), which belongs – as EPO – to the hematopoietin family of cytokines)[12] (an interaction between heparin and EPO would impose additional negative charges on EPO and thus might influence its electrophoretic mobility); and (4) heparin and other glycosaminoglycans (GAGs; e.g. heparan sulfate)[13] are also endogenously produced by the human body. Aside from the impact on IEF-PAGE the influence of heparin on the performance characteristics of SDS-PAGE was also investigated. SDS-PAGE is typically used during the confirmation procedure of rhEPO-positive doping control samples.

* *Correspondence to: Christian Reichel, Doping Control Laboratory, AIT Seibersdorf Laboratories, A-2444 Seibersdorf, Austria.*
E-mail: christian.reichel@seibersdorf-laboratories.at

Doping Control Laboratory, AIT Seibersdorf Laboratories, A-2444 Seibersdorf, Austria

Experimental

Materials

Acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne Ready-Sol IEF), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxidisulfate (APS), urea, tris(hydroxymethyl) aminomethane (Tris), glycine, and the support film for polyacrylamide gels (GelBond) were from GE Healthcare (Uppsala, Sweden). Carrier ampolytes (Servalytes 2–4, 4–6, and 6–8) were obtained from Serva (Heidelberg, Germany). Dithiothreitol (DTT), phosphate buffered saline (PBS; pH 7.4) tablets, sodium bisulfite, Tris buffered saline (TBS; pH 8.0), and Alcian blue 8GX were purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphoric acid (85%, p.a.), glacial acetic acid (p.a.), methanol (LiChrosolv, gradient grade), formic acid (98–100%, p.a.), acetonitrile (ACN; LiChrosolv, hyper grade) and LiChrosolv water were from Merck (Darmstadt, Germany).

Reference standards for human recombinant erythropoietin (rhEPO; BRP-EPO batch 3) and human urinary erythropoietin (uhEPO; second international reference preparation) were bought from the European Directorate for the Quality of Medicines (Strasbourg, France) and the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK), respectively. NeoRecormon (Roche, Mannheim, Germany) and Aranesp (NESP; Amgen, Thousand Oaks, CA, USA) were used as standards for rhEPO-pharmaceuticals. Lovenox® (enoxaparin sodium, 80 mg/0.8 mL; average M_r *ca* 4500 Da (<2000 Da: ≤20%, 2000–8000 Da: ≥68%, >8000 Da: ≤18%)[14]; Sanofi Aventis GmbH; Vienna, Austria) and Ivor® (bemiparin sodium; 2500 I.E/0.2 mL; average M_r *ca* 3600 Da (<2000 Da: <35%, 2000–6000 Da: 50–75%, >6000 Da: <15%)[15]; Gerot Pharmazeutika; Vienna, Austria) were used as LMWH, Depot-heparin Immuno® (25 000 I.E./mL; M_r *ca* 8000–25 000 Da; Ebewe Pharma Ges.m.b.H; Unterach, Austria)[16] and heparin from porcine intestinal mucosa (average M_r *ca* 15 000 Da; Sigma-Aldrich, St Louis, MO, USA) as unfractionated (high molecular weight) heparins (UFH). Heparan sulfate (HS) from bovine kidney and porcine intestinal mucosa were also purchased from Sigma-Aldrich.

NuPAGE BisTris polyacrylamide gels (10% T, 1.5 mm), lithium dodecyl sulfate (LDS) sample buffer (4×), 4-morpholine-propanesulfonic acid (MOPS) electrophoresis running buffer (20×), and molecular weight marker solutions (SeeBlue Plus2, Mark12) were provided by Invitrogen (Carlsbad, CA, USA).

Devices for micro- and ultrafiltration (Steriflip filters, 0.2 µm; Amicon Ultra-15 and Amicon Ultra-0.5, both with a nominal molecular weight limit (NMWL) of 30 kDa) and membranes for Western blotting (Durapore, Immobilon-P) were from Millipore (Billerica, MA, USA) and Pall (Ann Arbor, MI, USA; Nanosep MF, 0.2 µm), respectively, and the fold filters (595½) were bought from Whatman (Dassel, Germany). The EPO-purification kit was obtained from MAIIA Diagnostics (Uppsala, Sweden) and the vacuum manifold used in combination with the kit was from QIAGEN GmbH (Hilden, Germany; QIAvac system). Antibodies were purchased from R&D Systems (Minneapolis, MN, USA; clone AE7A5) and Pierce (Rockford, IL, USA; ImmunoPure goat anti-mouse IgG (H+L)); the streptavidin horseradish peroxidase complex was from Biospa (Milano, Italy). Western blots were done on a semi-dry blotter (Trans-Blot SD; BioRad, Hercules, CA, USA). Tween-80 (Surfact-Amps 80) and the substrate for enhanced chemiluminescence detection (West Pico) were purchased from Pierce, and the non-fat milk (NFM) used for blocking, washing, and incubation steps was from BioRad. A LAS-4000 CCD-camera (Fujifilm; Tokyo, Japan) was used for image acquisition and GASepo version 1.3b2 (ARC; Seibersdorf, Austria)[17] for all image analyses.

Samples

Urine samples were received from three healthy volunteers who gave their written consent to using the anonymous samples for research purposes. The samples were used as negative controls and for studying the effect of different heparins (LMWH, UFH) on IEF- and SDS-PAGE (spiking experiments). Two volunteers received a single dose application of 50 000 I.E. of UFH (Depot-heparin Immuno®) subcutaneously (*ca* 670 I.E./kg; for medicinal purposes typical UFH-doses range between 5000 and 25.000 I.E. and are applied every 8 to 12 h), one volunteer received 3000 IU of Dynepo (*ca* 35 IU/kg). Samples were collected three days before and up to six days after the injection. All urine samples were aliquoted and stored frozen at −20 °C until analysis. After thawing, the samples were supplemented with protease inhibitors (Complete; Roche; Mannheim, Germany) and pH adjusted to 7.4 with 3.75 M Tris-HCl buffer essentially as described by Lasne *et al*.[18] Insoluble precipitates and cells were removed by centrifugation (2700 *rcf*, 10 min) and microfiltration with Steriflip filters. The urines (typically 20 mL per sample) were then concentrated and diafiltered (50 mM Tris-HCl (pH 7.4) buffer) down to 20–40 µL using a two-step ultrafiltration procedure (Amicon Ultra-15 (4,000 *rcf*/30 min), Amicon Ultra-0.5 (14,000 *rcf*/*ca* 15 min)). Spiking of urines with heparins was done immediately after thawing and before all subsequent sample preparation steps.

Immunoaffinity purification of EPO

For the extraction of recombinant and endogenous erythropoietins from heparin containing urinary retentates, a commercial EPO-purification kit was used. The protocol of the kit was followed with modifications.[19] Briefly, between 10 and 40 µL of each ultrafiltration retentate were filled up to 1 mL with 50 mM TBS pH 8.0 and 10 µL of detergent aid were added. The diluted retentates were then filtered through 0.2 µm spin filters (14 000 *rcf*/2 min) in order to prevent the affinity-monoliths from clogging. The monoliths were first equilibrated with 1 mL of washing buffer (as supplied with the kit) and at a flow rate of 1 mL/min using a vacuum manifold, then the sample was sucked through the monolith, and finally the monolith was washed again with 1 mL of washing buffer. The extracted EPO was desorbed from the immobilized anti-EPO antibodies on the monolith with 50 µL of an acidic elution buffer (desorption buffer A supplemented with 1% (v/v) of detergent aid) and in spinning mode (1000 *rcf*/1 min). Finally, the eluate was neutralized with 5 µL of adjustment buffer (prepared according to the supplied protocol). For the subsequent SDS- or IEF-PAGE analysis 20–30 µL of the eluate were used.

IEF-PAGE of heparin and heparin containing EPO-samples and standards

Isoelectric focusing (IEF-PAGE) was performed on polyacrylamide slab gels (25 × 11.5 cm) containing 7 M urea and a 1 : 1 mixture of Servalytes 2–4 and 4–6 (4% (w/v) final carrier ampholyte concentration). Gels and electrolytes were prepared as already published.[18] The interelectrode distance was 10 cm, the gels were prefocused for 30 min at constant voltage (250 V), and then the samples were applied and focused for 3600 to 4000 Vh at constant current (25 mA). Gels with wells were cast instead

of using application pieces.[20] Four types of experiments were performed, namely IEF-PAGE of (1) LMWH, UFH, and heparan sulfates; (2) uhEPO and rhEPO standards spiked with increasing amounts of heparins; (3) urines spiked with heparins before sample preparation (i.e. before micro- and ultrafiltration); and (4) urines collected before and after subcutaneous application of UFH. Between 0.1 and 1 mg of LMWH (Lovenox®) and 0.25 and 100 I.E. of UFH (Depot-heparin Immuno®) were applied on the gel for studying the focusing behaviour of heparins. Dilutions were done with 50 mM Tris-HCl buffer (pH 7.4). Two types of heparan sulfates were investigated, i.e. heparan sulfate from bovine kidney and from porcine intestinal mucosa (0.5 mg each). After focusing and removal of the support film gels were stained with Alcian blue solution (experiment type 1). For the second type of experiments about 0.2 ng of BRP-EPO, NIBSC uhEPO, and NESP were spiked with increasing amounts of UFH (2.5 to 250 I.E. Depot-heparin Immuno®) or LMWH (12.5 I.E. Ivor®). Urinary retentates of rhEPO-negative and Dynepo-positive urines were supplemented with 25 and 250 I.E. of UFH (experiment type 3). During the first three days after the heparin application urine samples were collected for every 2 to 4 h for the fourth type of experiments, and then *ca* every 6 to 7 h for the next 3 days. Urinary retentates were heated for 3 min at 80 °C in order to inactivate proteases[18] or were saturated with solid urea and incubated for 60 min at room temperature with occasional vortexing and re-saturating with urea.[21] Tween-80 (1% (v/v)) was added before filling the retentates in the wells of the IEF-gel.

SDS-PAGE of heparin containing EPO-samples and standards

Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) of EPO-standards (NIBSC uhEPO, BRP rhEPO; 60–90 pg) containing 0, 10, or 25 I.E. UFH and immunoaffinity purified urinary retentates (*vide supra*) were performed as described elsewhere.[22] Briefly, samples were mixed with LDS buffer (1×) and denatured for 5 min at 95 °C under reducing conditions (100 mM DTT). MOPS running buffer was used as electrolyte. The catholyte was supplemented with 5 mM sodium bisulfite in order to prevent the sample from being re-oxidized during the run.[23] All electrophoretic separations were carried out at constant voltage (200 V) and for *ca* 55 min.

Alcian blue stain

After removal of the supporting-film IEF-gels were stained (without fixing) in a solution of 0.1% (w/v) Alcian blue 8GX in 25% (v/v) ethanol and 10% acetic acid (v/v).[24] After *ca* 2 h the gels were destained in 50% (v/v) methanol for 2–3 h. The Alcian blue staining solution was always freshly prepared and filtered through a 595 1/2 fold filter. Due to the highly negatively charged nature heparin has to be stained with a positively charged dye, e.g. Alcian blue – and not Coomassie-dyes, which are negatively charged themselves.

Western blot with chemiluminescence detection

IEF-PAGE gels were double-blotted as described by Lasne *et al.*[18] with slight modifications. Gels were first equilibrated for 2 min in Towbin buffer (25 mM Tris, 192 mM glycine) and then proteins were transferred to a polyvinylidene fluoride (PVDF; Immobilon-P) membrane under semi-dry conditions (constant current (1 mA/cm^2), 30 min). Immediately after the transfer the membrane was shortly washed in phosphate buffered saline (PBS) and then incubated for 60 min at 37 °C in a solution of DTT in PBS (5 mM). The membrane was again washed in PBS and then blocked in 5% (w/v) NFM/PBS for 60 min at room temperature. After another short PBS-wash the blot was incubated in a 1 µg/mL dilution of the primary antibody (clone AE7A5) in 1% (w/v) NFM/PBS, typically overnight and at 4–8 °C. Then the membrane was washed in 0.5% NFM/PBS (3 × 10 min), blotted semi-dry and under acidic conditions (0.7% acetic acid; 0.8 mA/cm^2) on a second PVDF-membrane, blocked again in 5% NFM/PBS (60 min, room temperature), and incubated in a 1 : 2000 (v/v) dilution of

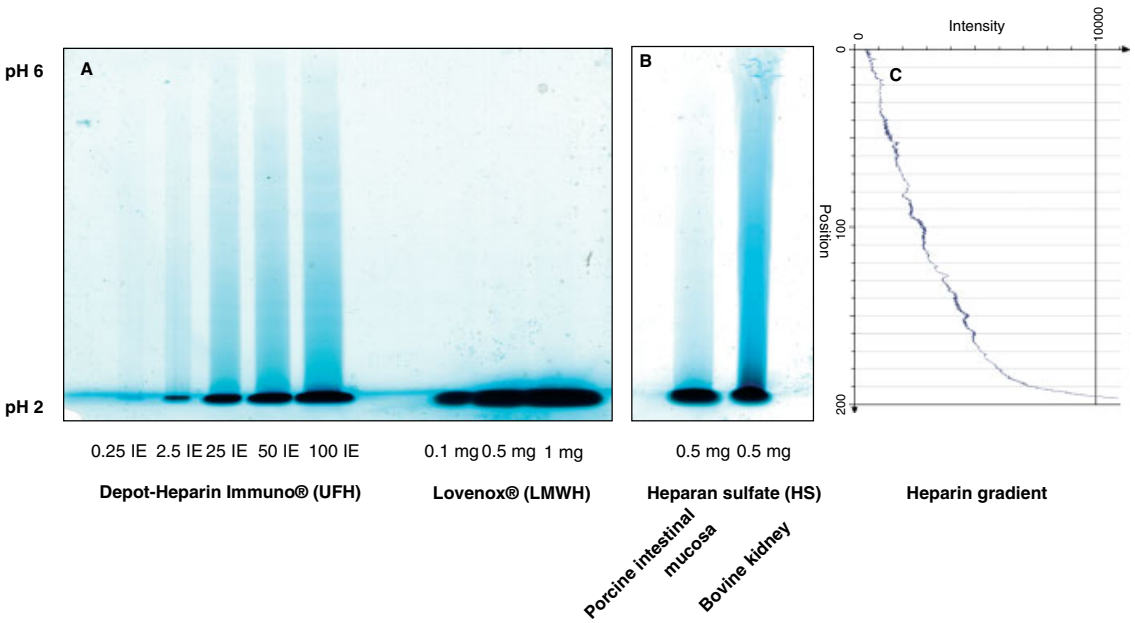

Figure 1. Migration behaviour of heparins and heparan sulfates on IEF-PAGE (Alcian blue stain). (A) shows the performance characteristics of LMWH (Lovenox®) and UFH (Depot-heparin Immuno®); (B) heparan sulfates (bovine kidney, porcine intestinal mucosa) - the formation of the heparin gradient is independent of the applied UFH-amount; (C) example of a gradient formed at 100 I.E.

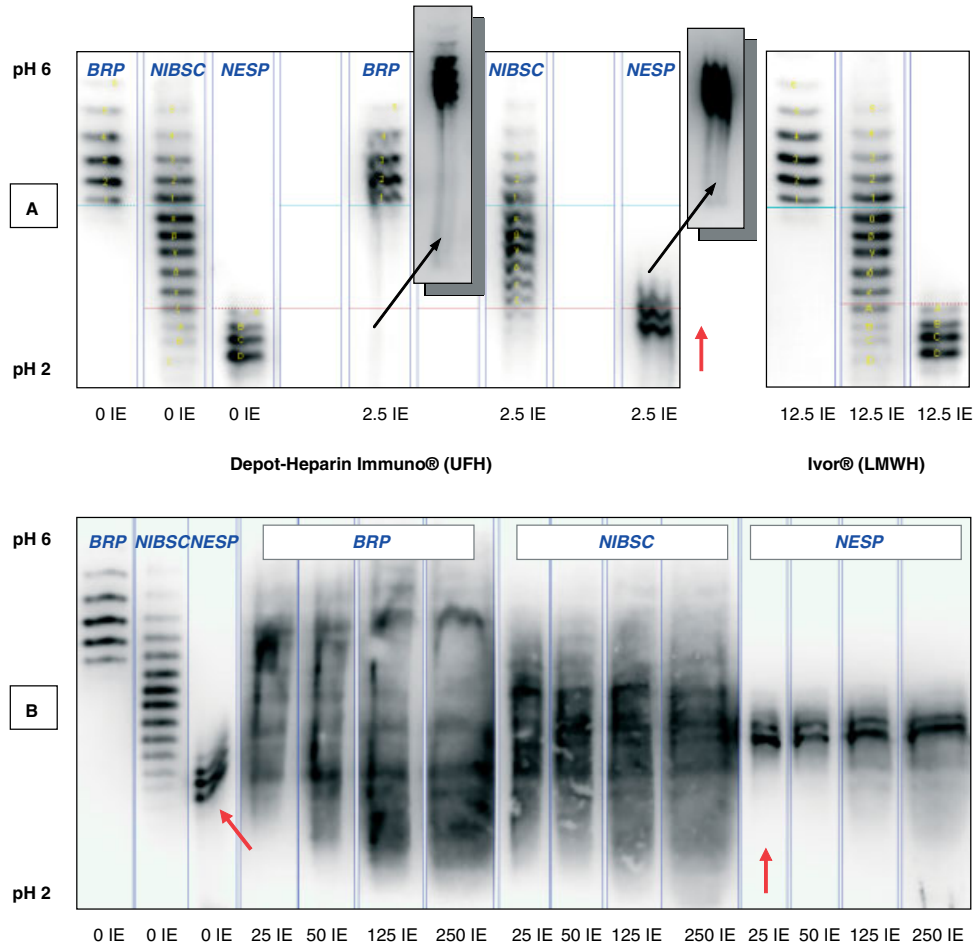

Figure 2. IEF-PAGE of EPO-standards spiked with an increasing amount of UFH and LMWH (western double-blot). At low UFH-concentrations bands became destabilized (wavy; (A) lanes 5, 7, and 9 from the left) and NESP-isoforms were slightly shifted towards the cathode ((A), lane 9 from the left, red arrow). Increased smearing was observed ((A) inserts with black arrows are lanes 5 and 9 but with enhanced contrast). No such effect was detectable for LMWH. Higher concentrations of UFH led to a loss of the focusing properties of the carrier ampholytes and to non-evaluable IEF-profiles (B). At these concentrations NESP resolved into a profile with only two discrete isoforms in the endogenous region of the gel ((B) lanes 1 to 4 from the right, red arrow).

the secondary goat anti-mouse IgG antibody in 1% NFM/PBS (60 min, room temperature). After three more washing steps (0.5% NFM/PBS; 3 × 10 min) and a 60 min incubation in streptavidin horseradish peroxidase (1 : 2000 (v/v), 1% NFM/PBS) the blot was washed in PBS (3 × 10 min) and developed with an enhanced chemiluminescence substrate (West Pico). The exposure time of the CCD-camera (LAS-4000) was adjusted according to the signal strength and ranged from seconds to minutes.

Western blots of SDS-PAGE gels were performed as already described elsewhere.[22] In brief, the gel was equilibrated in Bjerrum buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol; 3 × 5 min) and the proteins were blotted for 60 min on a PVDF-membrane (1 mA/cm^2). The remaining steps were identical to the steps described for IEF-PAGE gels.

Results and Discussion

IEF-PAGE of LMWH, UFH, and heparan sulfate

Low molecular weight heparins (Lovenox®, Ivor®) and unfractionated heparins (Depot-heparin Immuno®, heparin from porcine intestinal mucosa) behaved differently during focusing on pH 2–6 IEF-PAGE gels. Due to its polyanionic nature (i.e. the lack of an isoelectric point) both types of heparin should migrate completely into the anode.[9] However, only LMWH behaved as predicted and regardless of the concentration applied on the gel (Figure 1A). UFH, on the other hand, migrated only partly towards the anode and was spread over the entire pH-range of the gel – building up a continuous heparin gradient ('smear') from cathode to anode (Figures 1A and 1C) with the highest concentration at the anode. This behaviour was already observed by several scientists during the 1970s – the early days of carrier ampholyte electrophoresis. However, these early publications[25–27] presented up to 21 clearly focused 'heparin isoforms', which were later decoded as pure artefacts – namely as 21 different complexes of one heparin polymer with 21 different carrier ampholyte molecules. For these studies Ampholines were used. The EPO IEF-method uses Servalytes, which are chemically different from Ampholines. (Ampholines are polyamines with short side chains bearing terminal carboxylic groups, Servalytes are polyamines with side chains containing terminal phosphonate and sulfonate groups.[28] Whether these differences lead to differences in the interaction between UFH,

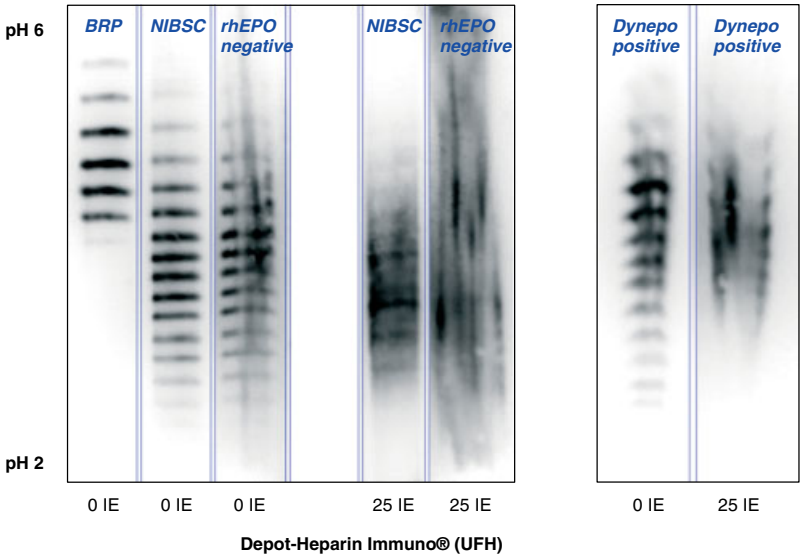

Figure 3. Influence of UFH on EPO (uhEPO, Dynepo) in urine and urinary retentates (western double-blot). Due to promoting excessive smearing on IEF-PAGE the heparin gradient made an evaluation of the EPO IEF-profiles impossible according to the technical document of WADA (TD2009EPO). Also note that the UFH-free retentates already show slight smeaing. This is probably due to endogenous GAGs present in these urines, an effect less pronounced on large sized (i.e. higher capacity) gels.

carrier ampholytes, and EPO was not part of this study.) It was also demonstrated that the interaction between carrier ampholyte and heparin molecules is strongly pH-dependant, roughly 10 000-fold higher at pH 3 than at pH 7.[29] Since the tested UFH were a complex mixture of heparin polymers no discrete isoforms were observable on our Alcian blue stained IEF-gels. Back then Righetti *et al*. showed that 'heparin isoforms' were only observable on gels containing no urea but not on gels containing 8 M urea.[9] No such effect was observable on our 7 M urea gels (the gels routinely used for rhEpo-testing) and also not if the urea concentration was increased or the rhEpo-standards were saturated with urea (by adding solid urea and incubating for 60 min at room temperature before applying the standards on the gel; data not shown). Again, we attribute this to the complex composition of the tested UFH (M_r *ca* 8000–25 000). The formation of this heparin gradient within the urea-containing IEF-gel was also independent of the UFH-concentration, i.e. no complete migration of the heparin molecules into the anode was observed as it was detected at the various LMWH-concentrations (Figure 1C).

Since heparin is chemically closely related to heparan sulfate (HS; the predominant uronic acid of the disaccharide repeating unit is glucuronic acid instead of iduronic acid, and the D-glucosamine amino sugar is less N-sulfated)[30] and since HS is a glycosaminoglycan (GAG) and – as heparin – naturally occurring in normal (non-diseased) urine, the behaviour of two different types of heparan sulfates on IEF-PAGE was also tested. Both HS showed identical migration characteristics as heparin – generating a continuous heparan sulfate gradient towards the anode (Figure 1B). HS is also part of the glomerular basement membrane (GMB) of the kidney.[31] Degradation of HS in the GBM does not necessarily lead to proteinuria,[32–33] which is frequently observed in 'effort urines'.[34–35]

Effect of heparins on IEF-PAGE of erythropoietin standards and urinary retentates

Standards for human recombinant erythropoietins (BRP-EPO, NESP) and human urinary EPO were spiked with increasing amounts of LMWH and UFH, and EPO-profiles were detected immunologically after western double-blot. At a concentration of 2.5 I.E. UFH all three standards showed slightly distorted IEF-profiles: (1) the isoforms were no longer straight but wavy (i.e. destabilized); and (2) showed smears (predominantly in the region below the most acidic isoform). NESP appeared to be most sensitive to UFH – a profound shift of the isoform cluster towards the endogenous region was observable (Figure 2A). At higher concentrations (25 to 250 I.E.) the IEF-profile was completely destroyed and no longer useful for isoform quantitation purposes (Figure 2B). And NESP was entirely shifted to the endogenous region. Only 2–3 discrete isoforms were observable while the remaining 3–4 isoforms vanished in the smear below the most acidic isoform. UFH also affected the separation of NESP when the NESP-standard was next to a lane containing UFH. In this case heparin caused that part of the isoforms which was nearest to the UFH-containing lane to be shifted towards the cathode while with increasing horizontal distance from the UFH-lane the NESP isoforms stayed at the expected pI-position (Figure 2B, third lane from the left). Similar experiments were performed with LMWH (Ivor®), but LMWH showed no influence on the IEF-profile of the various EPO-standards (Figure 2A). Consequently, the distortion of the EPO IEF-profile was due to the high molecular weight heparin molecules and was attributable to the heparin gradient which was formed by UFH in the pH 2–6 IEF-gel (culminating in the highest concentration of heparin molecules on the acidic (i.e. the NESP isoform-focusing) side of the gel). This gradient led to a shift of the most acidic EPO-isoforms towards the cathode.

Next, the influence of UFH on urinary retentates (obtained by ultrafiltration with 30 kDa NMWL filters) was investigated. The same behaviour as observed for EPO-standards was noticeable, i.e. the profiles of both the rhEPO-negative control urines and the rhEPO (Dynepo)-positive urines from an excretion study became non-evaluable after spiking UFH into the urine (Figure 3). Due to the strong 'smearing effect', which UFH had on the IEF-profiles, isoform-quantitation and image-evaluation as described in TD2009EPO[36] was no longer possible.

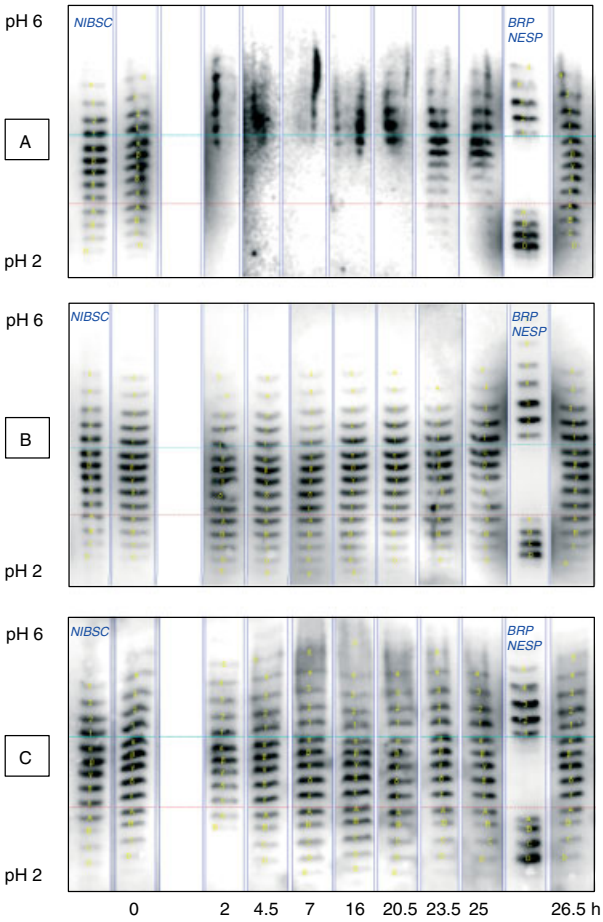

Figure 4. Results of an UFH-application study (western double-blot). The endogenous urinary EPO-profile completely or partly got abolished for *ca* 25 h after subcutaneous application of UFH (A). After immunoaffinity purification of the urinary retentates (B) or treatment with solid urea (C) EPO-isoforms became visible again.

Effect of UFH on IEF-PAGE of erythropoietins in urine samples

The result of the application study (a single dose of 50 000 I.E. of Depot-heparin Immuno® was applied to healthy persons) further confirmed the effect of UFH on EPO IEF-profiles, and that this effect was still observable after passage of UFH through the human body. Already 2 h after the application the endogenous EPO-profile was extinguished and gradually re-appeared after 23.5–26.5 h. Until 20.5 h after the injection no useful EPO-isoforms were detectable (Figure 4A). Consequently, UFH had a masking effect on EPO-isoforms for almost one day.

Remedies of the UFH-effect

IEF-PAGE after immunoaffinity purification

Purification of urinary retentates by immunoaffinity (clone 3F6 anti-Epo antibody immobilized on disposable monolithic columns; MAIIA Diagnostics) led to an efficient removal of UFH and consequently the harmful effect of UFH on EPO IEF-PAGE. Thus, the disappearance of the isoforms during the first 25 h after the application of UFH was indeed not due to a suppressive effect of high molecular mass heparins on the endogenous EPO-production but due to the heparin gradient on the IEF-gel. This also demonstrated that high molecular mass heparins can be excreted in urine without having been significantly metabolized by liver heparinase (Figure 4B).[37] However, slight 'smears' on EPO IEF-profiles are also occasionally observed in non-heparin-treated urine samples and are probably due to endogenous glycosaminoglycans, which naturally occur in human urine (e.g. chondroitin sulfate, heparan sulfate, dermatan sulfate).[38–39] Smearing may also be caused by faulty application pieces, which are typically used for applying urinary retentates on IEF-gels. This effect was attributable to a badly done impregnation in order to make the pieces easily wettable. Hence, we recommend casting gels with wells and entirely omit using application pieces[20].

IEF-PAGE after treatment with solid urea

Urea, a chaotropic agent, solubilizes proteins and breaks protein-protein interactions. The solubilizing and dissociating effect of urea is mainly due to the disruption and destabilization of hydrophobic and hydrogen bonds.[40] For unfolding proteins in solution urea concentrations between 4 and 9 M are necessary. The concentration typically used in IEF-polyacrylamide gels is 7 M. While no effect of urea was observable when performing IEF-PAGE of UFH (*vide supra*), the addition of solid urea to the urinary retentates obtained after subcutaneous administration of UFH – thus generating a saturated solution – successfully destabilized the interaction between EPO, heparin, and carrier ampholytes (Figure 4C). No such effect was observable when UFH without EPO was treated with saturated urea (data not shown). However, at least three aspects have to be considered when treating samples with urea: (1) during and after the treatment samples should be not heated over 37 °C as carbamylation of proteins may occur (thus leading to a shift of isoforms to the acidic pH-region);[41] (2) the temperature of the cooling unit used during focusing should be increased to e.g. 15 °C (instead of the usual 8–10 °C) because saturated urea solutions rapidly crystallize at low temperatures; and (3) urea leads to an increase in sample volume. The latter might hamper the application of the entire – now increased – volume on application pieces (which typically accept *ca* 20 µL). This drawback can again be circumvented by using gels with precast wells. In case of a then (i.e. after urea treatment) rhEPO-positive urinary sample an additional confirmation by SDS-PAGE according to WADA TD2009EPO should be performed.

SDS-PAGE after immunoaffinity purification

Due to the high protein content of urinary retentates after two ultrafiltration steps – usually in the µg/µL range (e.g. 10–40 µg/µL) – SDS-PAGE for the detection of EPO routinely requires an immunoaffinity extraction step before the samples can be applied on the gel. Otherwise the gel would be overloaded and bands distorted. Immunoaffinity purification of EPO can be achieved, for example, via ELISA,[22,42] monolithic disks,[19] magnetic beads,[43] or column chromatography.[44] Due to convective mass transfer instead of diffusion mass transfer monolithic disks allow immunoaffinity extractions within minutes and without cross contamination (single usage devices).[45] The monolithic immunoaffinity devices used in this study allowed EPO-enrichment within a few minutes and with high apparent recoveries (*ca.* 65%).[46] SDS-PAGE itself appeared to be inert against an interference of UFH on the separation of EPO standards (Figure 5A): heparin concentrations which profoundly affected the separation of the various EPOs on IEF-PAGE (e.g. 25 I.E.) had no influence on the separation by SDS-PAGE. Consequently, immunoaffinity

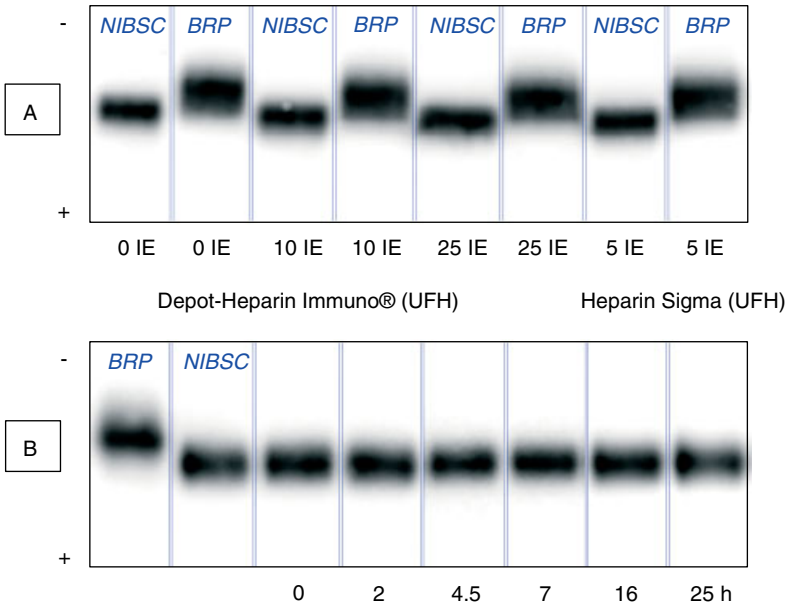

Figure 5. SDS-PAGE of EPO standards and urinary retentates containing heparin (western double-blot). UFH had no influence on the electrophoretic separation of the standards (A) and retentates obtained after application of UFH (B). However, due to the high protein content retentates have to be always cleaned up by immunoaffinity before application on gel.

purification of urinary retentates was only necessary in order to reduce the high protein content of the retentates but not for abolishing a possible heparin effect. Consequently, even if no immunoaffinity purification is done for IEF-PAGE an abuse of rhEPO can be detected by SDS-PAGE – regardless of whether UFH was used or not. Hence, we recommend to routinely confirm suspicious IEF-profiles by SDS- and SARCOSYL-PAGE (the latter especially on spec of MIRCERA-abuse).[22] Figure 5B shows the results obtained after immunoaffinity purification of retentates of a UFH application study. No influence of heparin on the analysis result was detectable.

Conclusion

Due to their highly charged nature and high molecular mass, UFH and GAGs can act destructive on the EPO IEF-PAGE method – the method routinely used for detecting doping with recombinant erythropoietins. Namely, (1) the EPO-profile can get *smeared* or *completely abolished* (smeared profiles do not fulfil the acceptance criteria of WADA TD2009EPO); (2) the NESP-profile can get *shifted* towards or into the endogenous area; (3) the degree of the NESP-shift is dependant on the heparin concentration; and (4) rhEPO-positive profiles can become invalid due to excessive smearing. The described effects are only observable for UFH but not for LMWH. UFH forms a heparin gradient on the 7 M urea IEF-gel with increasing heparin concentration towards the anode. Consequently, the effects are most intense in the acidic region (NESP-region) where EPO-isoforms can become shifted towards the endogenous region. In the endogenous and basic region – where the heparin concentration is lower – the EPO-profile gets smeared or completely abolished. The impact of UFH is independent of the sample type (standards, urine, and urinary retentates) and can be observed in samples spiked with heparin or in urine after subcutaneous application of high doses of UFH. The extent of the impact is strongly concentration dependant and only observable for IEF-PAGE but not for SDS-PAGE. Successful remedies are the saturation of urinary retentates with urea before application on the IEF-gel, immunoaffinity purification of retentates (or urine) before performing IEF-PAGE, or to routinely perform an SDS-PAGE on all suspicious samples, especially if no immunoaffinity cleanup was made before IEF-PAGE.

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References

[1] A. Carlsson, in *Essential Guide to Blood Coagulation*, (Eds: M. Blombäck, J. P. Antovic), Wiley-Blackwell: Chichester, UK, **2010**, pp. 67–90.
[2] B. Kemkes-Matthes, G. Oehler, *Blutgerinnung und Thrombose*, Georg Thieme Verlag: Stuttgart, **2001**, pp. 3–5.
[3] D. Hoppensteadt, O. Iqbal, J. Fareed, in *Chemistry and Biology of Heparin and Heparan Sulfate*, (Eds: H. G. Garg, R. J. Linhardt, C. A. Hales), Elsevier: Amsterdam, **2005**, pp. 583–606.
[4] T. T. Horlocker, J. A. Heit, *Anesth. Analg.* **1997**, *85*, 874.
[5] B. Casu, in *Chemistry and Biology of Heparin and Heparan Sulfate*, (Eds: H. G. Garg, R. J. Linhardt, C. A. Hales), Elsevier: Amsterdam, **2005**, pp. 1–28.
[6] S. A. Mousa, in *Chemistry and Biology of Heparin and Heparan Sulfate*, (Eds: H. G. Garg, R. J. Linhardt, C. A. Hales), Elsevier: Amsterdam, **2005**, pp. 571–581.
[7] G. Hiendlmayer, *Gerinnungshemmer*, Kohlhammer: Stuttgart, **2007**, pp. 32–33.
[8] H.-J. Schäfer, W. Schänzer, U. Schwabe, *Abschlussbericht der Expertenkommission zur Aufklärung von Dopingvorwürfen gegenüber Ärzten der Abteilung Sportmedizin des Universitätsklinikums Freiburg*, Stabsstelle Presse- und Öffentlichkeitsarbeit Universitätsklinikum Freiburg, Cologne, **2009**, pp. 15–16.
[9] P. G. Righetti, A. V. Stoyanov, M. Y. Zhukov, *The Proteome Revisited*, Elsevier: Amsterdam, **2001**, pp. 162–164.
[10] I. Capila, R. J. Linhardt, *Angew. Chem. Int. Ed.* **2002**, *41*, 390.
[11] H. E. Conrad, *Heparin-Binding Proteins*, Academic Press: San Diego, **1998**, pp. 421–422.

[12] J. J. Oppenheim, M. Feldmann, in *Cytokine Reference* (Eds: J. J. Oppenheim, M. Feldman, S. K. Durum, T. Hirano, J. Vilcek, N. A. Nicola), Academic Press: San Diego, **2001**, p. 9.
[13] F. Zhang, Z. Zhang, R. J. Linhardt, in *Handbook of Glycomics*, (Eds: R. D. Cummings, J. M. Pierce), Academic Press: Amsterdam, **2009**, pp. 59–80.
[14] Sanofi-Aventis, *Lovenox® Prescribing Information*, **2009**, Available at: http://products.sanofi-aventis.us/lovenox/lovenox.html [27 June 2010].
[15] Gerot Pharmazeutika, *Ivor® 2500 I.E. Prescribing Information*, **2006**, Available at: www.pharmazie.com/graphic/A/71/1-24671.pdf [27 June 2010].
[16] Ebewe Pharma, *Depot-Heparin Immuno® Prescribing Information*, **2004**, Avialable at: http://www.baxter.at/downloads/aerzte_und_patienten/fachinfo/fi_heparin_immuno_25000ie_dezember 2004.pdf [27 June 2010].
[17] I. Bajla, I. Holländer, M. Minichmayr, G. Gmeiner, C. Reichel, *Comput. Methods Programs Biomed.* **2005**, *80*, 246.
[18] F. Lasne, L. Martin; N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*, 119.
[19] MAIIA Diagnostics, in *EPO Purification Kit Directions for Use* (0250), Uppsala, **2010**, Available at: http://www.maiiadiagnostics.com/files/Directions%20for%20Use%20-%20EPO%20Purification%20Kit.pdf [27 June 2010].
[20] R. Westermeier, *Electrophoresis in Practice*, Wiley-VCH Verlag: Weinheim, **2005**, pp. 14, 178.
[21] D. M. Bollag, M. D. Rozycki, S. J. Edelstein, *Protein Methods*, Wiley-Liss: New York, **1996**, p. 180.
[22] C. Reichel, F. Abzieher, T. Geisendorfer, *Drug Test. Analysis* **2009**, *1*, 494.
[23] Invitrogen, in *NuPAGE Technical Guide* Version E (IM-1001), Carlsbad, **2003**, pp. 12–16.
[24] R. P. Estrella, J. M. Whitelock, R. H. Roubin, N. H. Packer, N. G. Karlsson, in *Methods in Molecular Biology, Glycomics: Methods and Protocols, Vol. 534*, (Eds: N. H. Packer, N. G. Karlsson), Humana Press: New York, **2009**, pp. 171–192.
[25] N. M. McDuffie, C. P. Dietrich, H. B. Nader, *Biopolymers* **1975**, *14*, 1473.
[26] C. P. Dietrich, H. B. Nader, N. M. McDuffie, *An Acad. Bras. Cienc.* **1975**, *47*, 301.
[27] E. A. Johnson, B. Mulloy, *Carbohydr. Res.* **1976**, *51*, 119.
[28] T. Berkelman, in *Handbook of Isoelectric Focusing and Proteomics*, (Eds: D. Garfin, S. Ahuja), Elsevier: Amsterdam, **2005**, pp. 73–75.
[29] P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier: Amsterdam, **1983**, pp. 308–311.
[30] R. K. Murray, F. W. Keeley, in *Harper's Illustrated Biochemistry* (Eds: K. Murray, D. K. Granner, P. A. Mayes, V. W. Rodwell), McGraw-Hill: New York, **2003**, p. 545.
[31] B. Heintz, G. Stöcker, C. Mrowka, U. Rentz, H. Melzer, E. Stickeler, H. G. Sieberth, H. Greiling, H. D. Haubeck, *Hypertension* **1995**, *25*, 399.
[32] T. J. Wijnhoven, J. F. Lensen, R. G. P. Wismans, M. Lamrani, L. A. Monnens, R. A. Wevers, A. L. Rops, J. van der Vlag, J. H. Berden, L. P. van den Heuvel, T. H. van Kuppevelt, *J. Am. Soc. Nephrol.* **2007**, *18*, 823.
[33] T. J. Wijnhoven, J. F. Lensen, R. G. P. Wismans, D. J. Lefeber, A. L. Rops, J. van der Vlag, J. H. Berden, L. P. van den Heuvel, T. H. van Kuppevelt, *J. Am. Soc. Nephrol.* **2007**, *18*, 3119.
[34] F. Lasne, in *Pharmacology, Doping and Sports*, (Ed: J. L. Fourcroy), Routledge: London, **2009**, pp. 107–124.
[35] S. Lamon, L. Martin, N. Robinson, M. Saugy, J. Ceaurriz, F. Lasne, *Clin. J. Sport. Med.* **2009**, *19*, 311.
[36] World Anti-Doping Agency, **2009**, 1–9. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2009EPO_EN.pdf [27 June 2010].
[37] L. M. Hiebert, S. M. Wice, T. Ping, *J. Lab. Clin. Med.* **2005**, *145*, 151.
[38] E. Y. Lee, S. H. Kim, S. K. Whang, K. Y. Hwang, J. O. Yang, S. Y. Hong, *Am. J. Nephrol.* **2003**, *23*, 152-.
[39] F. Maccari, D. Gheduzzi, N. Volpi, *Clin. Chem.* **2003**, *49*, 380.
[40] Pharmacia Fine Chemicals, *Isoelectric Focusing: Principles and Methods*, Pharmacia Fine Chemicals AB: Uppsala, **1982**, pp. 92–93.
[41] J. McCarthy, F. Hopwood, D. Oxley, M. Laver, A. Castagna, P. G. Righetti, K. Williams, B. Herbert, *J. Proteome Res.* **2003**, *2*, 239.
[42] M. Kohler, C. Ayotte, P. Desharnais, U. Flenker, S. Lüdke, M. Thevis, E. Völker-Schänzer, W. Schänzer, *Int. J. Sports Med.* **2008**, *29*, 1.
[43] A. Lüdke, S. Lüdke, E. Völker-Schänzer, W. Schänzer, in *Recent Advances in Doping Analysis (17),* (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sportverlag Strauß: Köln, **2009**, pp. 301–304.
[44] F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Int. J. Biol. Macromol.* **2007**, *41*, 354.
[45] M. Schuste, E. Wasserbauer, A. Neubauer, A. Jungbauer, *Bioseparation* **2000**, *9*, 259.
[46] MAIIA Diagnostics, in *EPO Purification Kit for urine and serum specimens*, Uppsala, **2010**, Available at: http://www.maiiadiagnostics.com/files/EPO%20purification%20kit%20for%20urine%20and%20serum%20specimens.pdf [27 June 2010].