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HEPARIN: More than an anticoagulant - interference of heparin on IEF-PAGE of erythropoietins

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Abstract

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).

Results and Discussion

1. 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.[1] 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 (Figure 1A, 1C) with the highest concentration at the anode. Since the tested UFH were a complex mixture of heparin polymers no discrete isoforms were observable on our Alcian blue stained IEF-gels. 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)[2] 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.[3] Degradation of HS in the GBM does not necessarily lead to proteinuria[4-5], which is frequently observed in "effort urines".[6-7]

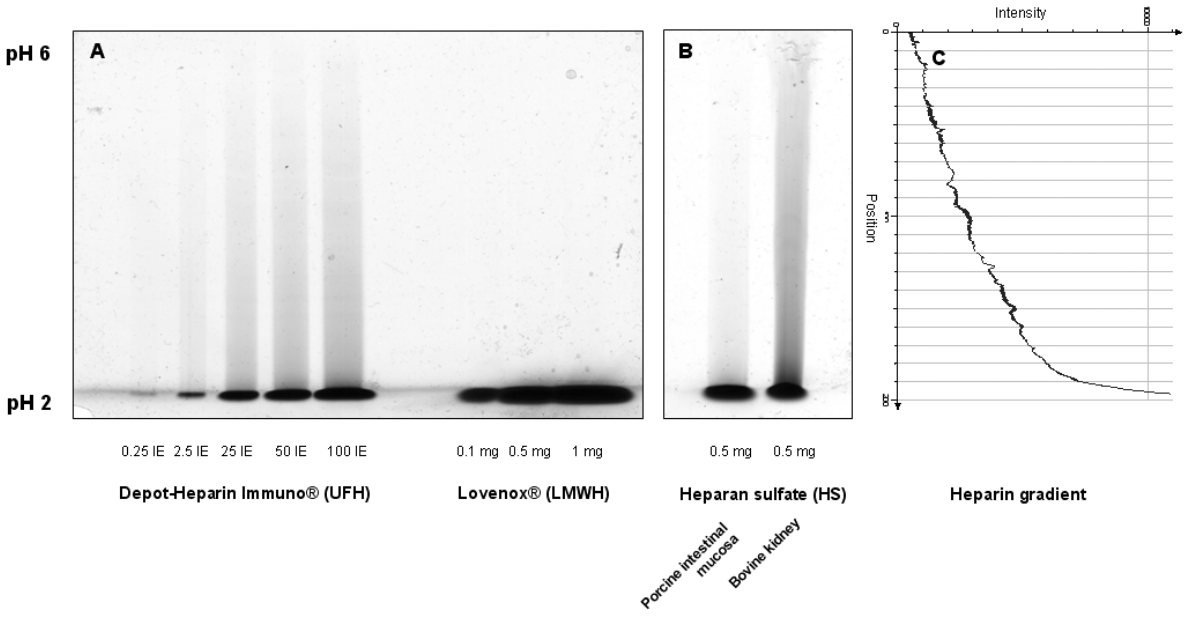

Figure 1. Migration behaviour of heparins and heparan sulfates on IEF-PAGE (Alcian blue stain). Figure 1A shows the performance characteristics of LMWH (Lovenox®) and UFH (Depot-heparin Immuno®), and Figure 1B of heparan sulfates (bovine kidney, porcine intestinal mucosa). The formation of the heparin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

2. 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. At higher concentrations (25 to 250 I.E.) the IEF-profile was completely destroyed and no longer useful for isoform quantitation purposes. 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. Similar experiments were performed with LMWH (Ivor®), but LMWH showed no influence on the IEF-profile of the various EPO-standards. 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 2). Due to the strong “smearing effect”, which UFH had on the IEF-profiles, isoform-quantitation and image-evaluation as described in TD2009EPO[8] was no longer possible.

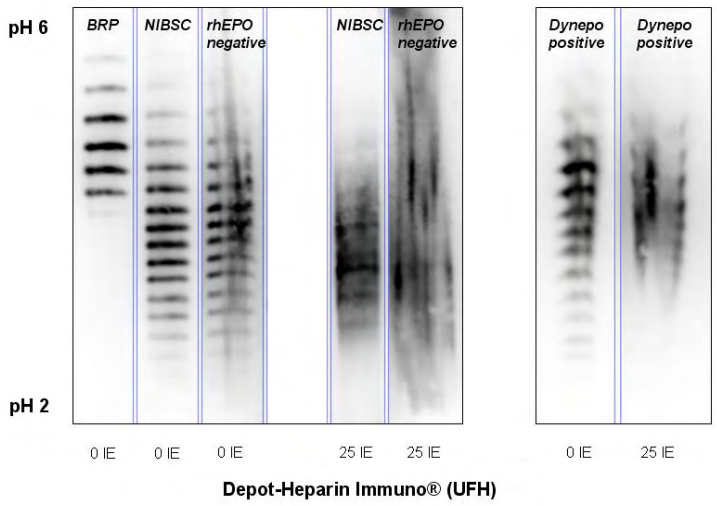

Figure 2. Influence of UFH on EPO (uhEPO, Dynepo) in urine and urinary retentates (Western double-blot). Due to promoting excessive smearing on IEF-PAGE the heparin gradient made an evaluation of the EPO IEF-profiles impossible according to the technical document of WADA (TD2009EPO). Also note that the 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.

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

The result of the application study (a single dose of 50000 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 two hours after the application the endogenous EPO-profile was extinguished and gradually re-appeared after 23.5-26.5 hours. Until 20.5 hours after the injection no useful EPO-isoforms were detectable (Figure 3A). Consequently, UFH had a masking effect on EPO-isoforms for almost one day.

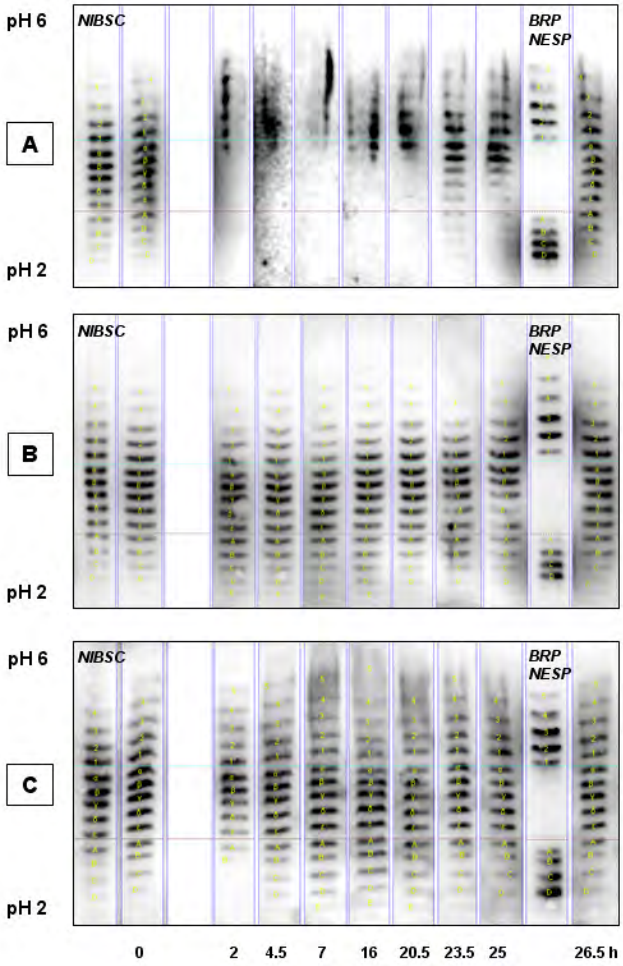

Figure 3. Results of an UFH-application study (Western double-blot). The endogenous urinary EPO-profile completely or partly got abolished for ca 25 hours after subcutaneous application of UFH (Figure 3A). After immunoaffinity purification of the urinary retentates (Figure 3B) or treatment with solid urea (Figure 3C) EPO-isoforms became visible again.

4. Remedies of the UFH-effect

4.1 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 hours 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 3B).[9] 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).[10-11] 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 (see also special article on practicing EPO IEF-PAGE, in this issue).

4.2 IEF-PAGE after treatment with solid urea: Urea, a chaotropic agent, solubilizes proteins and breaks protein-protein interactions. While no effect of urea was observable when performing IEF-PAGE of UFH, 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 3C). 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)[12], (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.

4.3 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 e.g. via ELISA[13-14], monolithic disks[15], magnetic beads[16], or column chromatography.[17] The monolithic immunoaffinity devices used in this study allowed EPO-enrichment within a few minutes and with high apparent recoveries (*ca* 65%).[18] SDS-PAGE itself appeared to be inert against an interference of UFH on the separation of EPO standards (Figure 4A): 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 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. Even if no immunoaffinity purification is done for IEF-PAGE an abuse of rhEPO can be detected by SDS-PAGE – regardless 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).[13] Figure 4B shows the results obtained after immunoaffinity purification of retentates of a UFH application study. No influence of heparin on the analysis result was detectable.

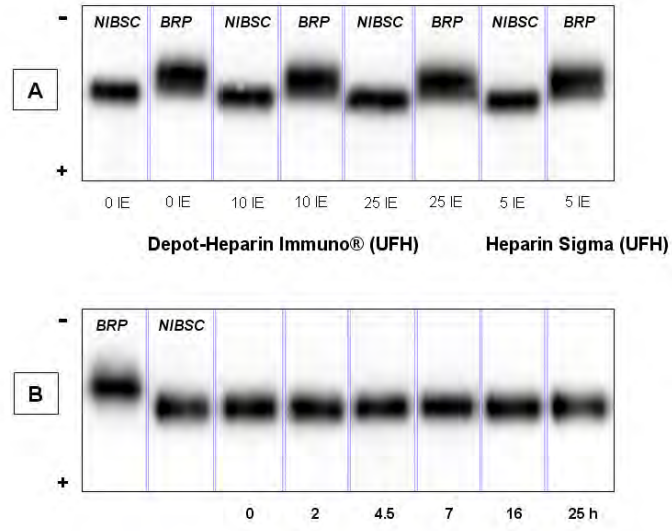

Figure 4. SDS-PAGE of EPO standards and urinary retentates containing heparin (Western double-blot). UFH had no influence on the electrophoretic separation of the standards (Figure 4A) and retentates obtained after application of UFH (Figure 4B). However, due to the high protein content retentates have to be always cleaned up by immunoaffinity before application on gel.

Note:

For additional details please refer to reference [19].

Acknowledgements

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