Philippe Desharnais, Jean-François Naud, Christiane Ayotte

Desialylation improves the detection of recombinant erythropoietins in urine samples analyzed by SDS-PAGE

Laboratoire de contrôle du dopage, INRS-Institut Armand-Frappier, Laval (Québec), Canada

Abstract

Recombinant erythropoietin (rhEPO) has been misused for over two decades by athletes, mainly but not only in endurance sports. A direct rhEPO detection method in urine by isoelectric focusing (IEF) was introduced in 2000 [1], but the emergence of “third generation” of erythropoiesis-stimulating agents and so-called “biosimilars” rhEPOs, together with the sensitivity of human endogenous EPO (huEPO) pattern to enzymatic activities and its modification following short strenuous exercise, prompted the development of a complementary test based on SDS-PAGE analysis [2-4]. While Mircera and NESP can be detected with the existing IEF and SDS-PAGE methods, some samples containing both epoetin-α/β and huEPO present profiles that may still be difficult to interpret. As doping practices having moved to microdosing, these mixed patterns are more frequently observed. We have investigated the impact of enzymatic desialylation on the urinary and serum EPO profiles obtained by SDS-PAGE with the aim of improving the separation of the bands in these mixed EPO populations. We have observed that the removal with neuraminidase of the sialic acid moieties from the different EPOs studied reduced their apparent molecular weight (MW) and increased the migration distance between huEPO and rhEPO centroids, therefore eliminating the size overlaps between them and improving the detection of rhEPO.

Introduction

Samples collected at the beginning or the end of microdoses excretion, resulting in mixed patterns of epoetin-α/β and huEPO, will generate SDS-PAGE profiles composed of a single widened band averaging the masses of all EPO isoforms without clear resolution. Reichel *et al.* reported structural differences between hEPO isolated from urine, serum or plasma and rhEPO [4] in agreement with the earlier report from Kung *et al.* [5] indicating that desialylation increases the mass differences between huEPO and rhEPO. It was also shown that desialylation removes the size overlap between epoetin-α/β and huEPO isoforms on 2D-PAGE analysis [6]. This study was undertaken to evaluate if desialylation may improve the detection of rhEPO by SDS-PAGE analysis, particularly in samples containing mixed equivalent populations.

Experimental

Material and Methods

huEPO (NIBSC, Berkshire, UK), epoetin α/β (BRP, European Pharmacopoeia Commission, Strasbourg, France), darbepoetin-α (NESP, Amgen, Thousand Oaks, CA), epoetin-δ (Dynepo, kind gift from Dr. C. Reichel, Seibersdorf, Austria). Monoclonal antibodies, clones 9C21D11 and AE7A5, were purchased from RnD Systems (Minneapolis, MN), human erythropoietin ELISA immunoassay kit from Stem Cell Technologies (Vancouver, BC) and Mircera ELISA SA-coated microplates from MicroCoat Biotechnologie GmbH (Bernried, Germany). Anti-mouse-biotin, streptavidin-HRP, anti-mouse HRP and Supersignal West Femto were purchased from Pierce (Rockford, IL). Ampholytes, pH 2-4, 4-6, 6-8, were obtained from Serva (Heidelberg, Germany). SDS-PAGE was performed on homecast Bis-Tris gels (10% T, 3%C), 1mm thickness with MOPS XT running buffer. Acrylamid and XT buffer were from Bio-Rad (Hercules, CA). Desialylation was done using neuraminidase (α2→3 > α2→8 = α2→6) from clostridium perfrigens (Roche, Laval, Canada). All other products were purchased from Sigma (St-Louis, IL).

 ISBN 978-3-86884-038-4

Sample preparation
Immunopurification of huEPO was performed on ELISA immunoassay plates, as already described [3]. Isolation of serum EPO (hsEPO) was first done on an Affi-Gel Hz hydrazide gel coupled to the anti-human EPO monoclonal antibody clone 9C21D11, according to a method previously described [7] and further purified on the anti-EPO plates from the Mircera ELISA kit.

Desialylation of EPOs reference standards
For reference standards and mixtures, each preparation (10 µL) was supplemented with 5 µL of phosphate buffer 20 mM pH 7.4 containing 5 mU of neuraminidase and incubated 1 h at room temperature. Longer incubation time at room temperature or at 37°C did not result in additional weight loss when analysed on SDS-PAGE and the reaction was determined to be completed in these conditions. Then, 15 µL of XT sample buffer 2X was added and the samples boiled for 3 min at 95°C before electrophoresis. Desialylation of purified urinary or serum EPO was performed on immunoassay plates by adding 100 µL of phosphate buffer 20 mM pH 7.4 containing 5 mU of neuraminidase, followed by an incubation for 1 h at room temperature. Wells were washed 3 times with PBS 1X, then 30 µL of XT sample buffer 1X were added and heated at 95°C for 3 min for EPO before SDS-PAGE.

Results and Discussion

Analysis of reference standards
The SDS-PAGE results for the intact (lanes 1 to 4) and desialylated (lanes 5 to 8) standards of erythropoietins tested are shown at Figure 1.

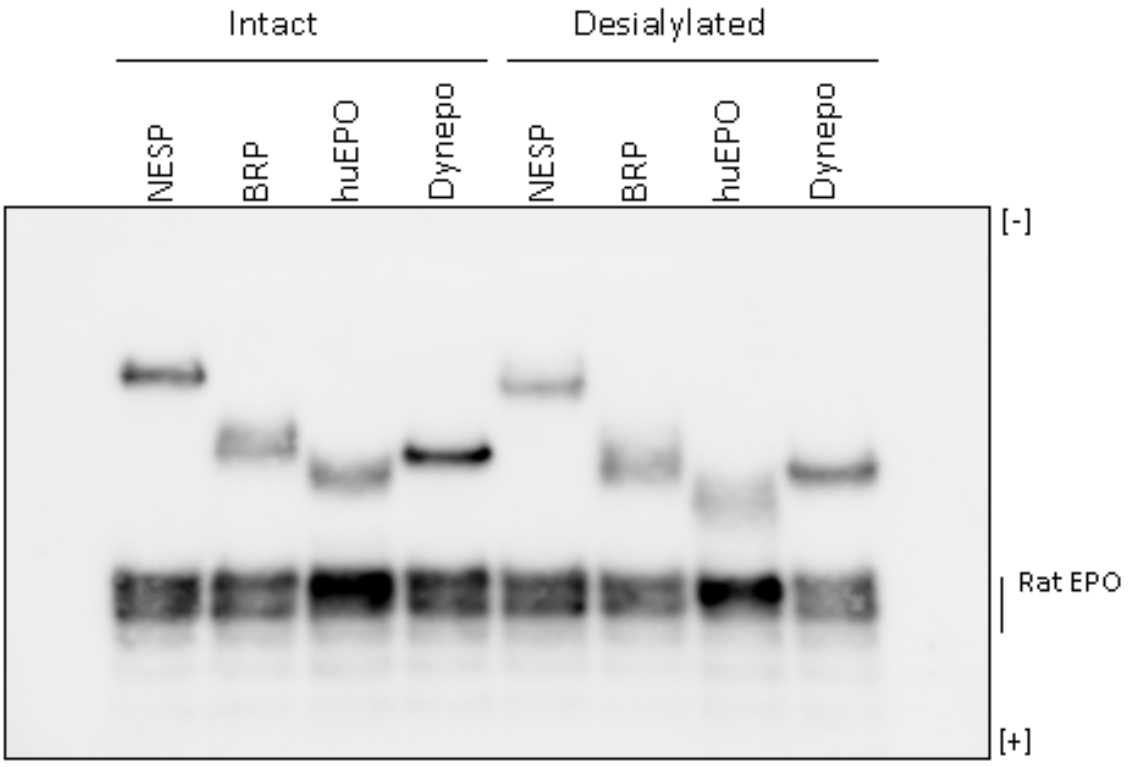

Figure 1: Analysis of EPOs standards by SDS-PAGE with and without treatment with neuraminidase: 0.05 ng darbepoetin-α, 10 mIU epoetin-α/β, 10 mIU huEPO and 10 mIU epoetin-δ analysed by SDS-PAGE before and after treatment with neuraminidase. Rat EPO (0.5 ng) was added in all lanes before loading as a migration control.

ISBN 978-3-86884-038-4

Consistent with its difference in sialic acids, the apparent MW of huEPO appears to be reduced more significantly than all the other rhEPOs analyzed [8,9]. Desialylation does not change the migration behavior, which remains diffuse for epoetin-α/β and huEPO and sharp for epoetin-δ and darbepoetin-α therefore suggesting that the heterogeneity originating from the N-antennary structures is responsible for the band shape on SDS-PAGE.

With classical IEF analysis, as shown in figure 2 and 3, it is only from equimolar concentrations of rhEPO α/β and huEPO or higher that the full WADA TD2009EPO [10] identification criteria are met (Fig. 2A, lane 5), while for epoetin-δ, it requires a ratio of epoetin-δ: huEPO equal to 2:1 (Fig. 3A, lane 6).

The detection of all recombinant epoetins on SDS-PAGE was improved by desialylation as shown in Figures 2C and 3C. In fact, a distinct band was obtained for epoetin-α/β and epoetin-δ in relative ratios to huEPO of respectively 0.5 (Fig. 2C, lane 3) and 0.25 (Fig. 3C, lane 2).

Athletes' samples showing atypical "effort-like" IEF profiles:
Athletes' urine samples found to possess IEF EPO profiles with more than 50% of basic isoforms were re-analysed with SDS-PAGE following treatment with neuraminidase. Typical results are found at Figure 4A to 4C (lanes 3 and 4).

As shown in figure 4B, under the SDS-PAGE conditions the urinary EPO from "effort-like" IEF profiles clearly co-localizes with the huEPO standard (Fig. 4B, lanes 3-4) as demonstrated before [4], confirming its endogenous origin. This is also the case for hsEPO (Fig. 4B, C lane 2) that cannot be confused with rhEPO. Interestingly, once desialylated hsEPO migrates to an intermediate position between huEPO and Dynepo (Fig. 4C, lane 2), which is also observed for the "effort-like" profiles presented (Fig 4C, lanes 3 and 4). The lane 2 shows also an unspecific band coming from the different immunopurification procedure performed for this sample (see sample preparation). The origin of this unspecific band is currently under investigation.

Conclusions

The presence of rhEPO α, β and δ along with huEPO can be more difficult to detect by the current IEF and SDS-PAGE analyses. By increasing the migration distance between huEPO and rhEPO, the desialylation facilitates the interpretation of the SDS-PAGE results. Atypical urinary EPO profiles that are thought to be induced by strenuous exercise and that may contain serum EPO isoforms, were found to be easier to interpret following desialylation. In these conditions, the removal of the sialic acids successfully eliminated the size overlap of isoforms in standards of huEPO and rhEPO and therefore, could be employed to help discriminating atypical profiles of endogenous origin from those excreted following the administration of microdoses of rhEPO.

ISBN 978-3-86884-038-4

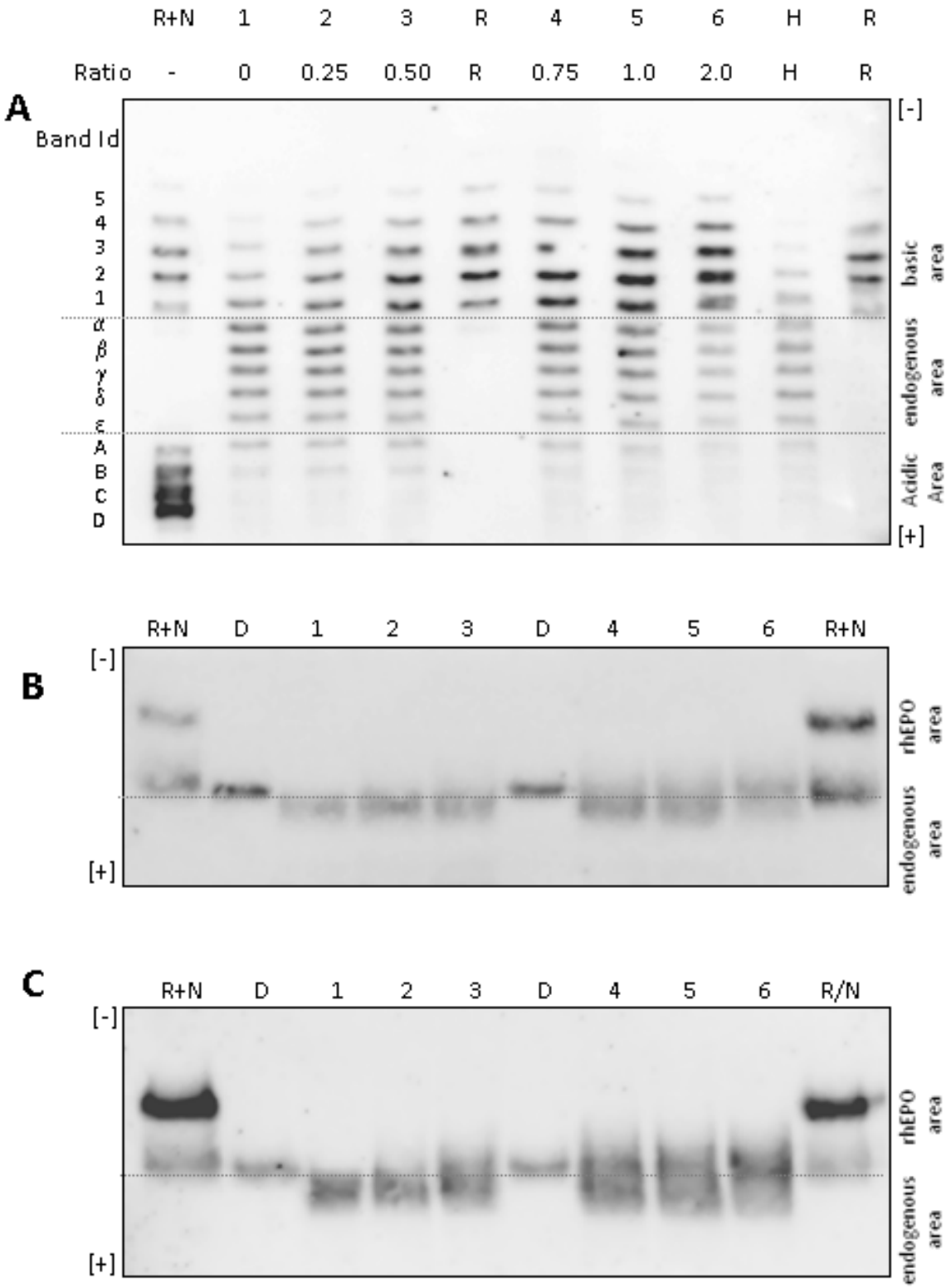

Figure 2: Analysis of epoetin-α/β and huEPO mixtures by IEF and SDS-PAGE with and without treatment with neuraminidase: huEPO standard was spiked with epoetin-α/β in ratios of 0 (lane 1), 0.25 (lane 2), 0.50 (lane 3), 0.75 (lane 4), 1.0 (lane 5) and 2.0 (lane 6) and analyzed by A) IEF; B) SDS-PAGE and C) SDS-PAGE with neuraminidase treatment (R: epoetin-α/β; N: darbepoetin; H: huEPO and D: epoetin-δ.)

 ISBN 978-3-86884-038-4

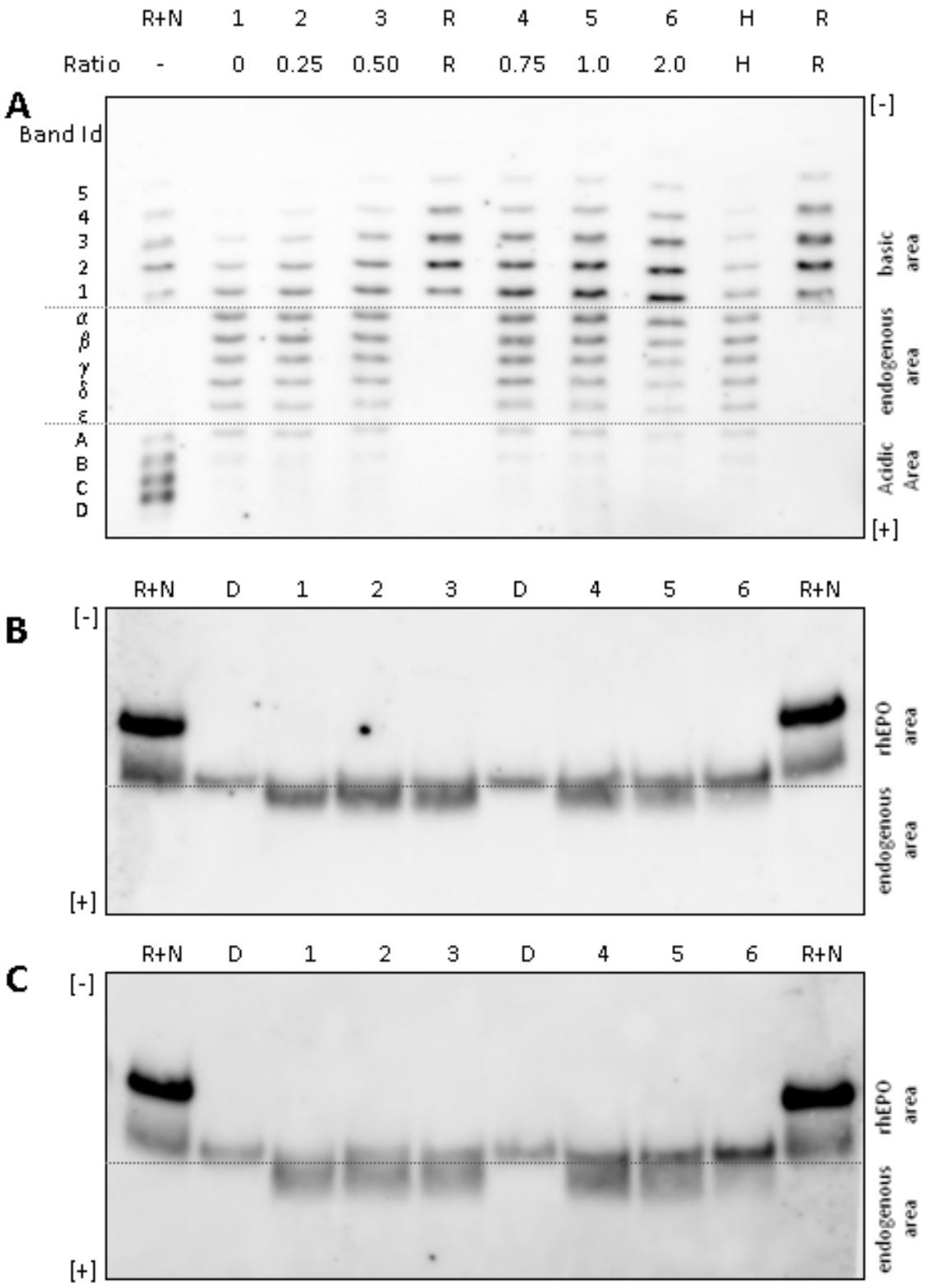

Figure 3: Analysis of epoetin-δ and huEPO mixtures by IEF and SDS-PAGE with and without treatment with neuraminidase: huEPO standard was spiked with epoetin-δ in ratios of 0 (lane 1), 0.25 (lane 2), 0.50 (lane 3), 0.75 (lane 4), 1.0 (lane 5) and 2.0 (lane 6) and analyzed by A) IEF; B) SDS-PAGE and C) SDS-PAGE with neuraminidase treatment (R: epoetin-α/β; N: Darbepoetin; H: huEPO and D: epoetin-δ).

ISBN 978-3-86884-038-4

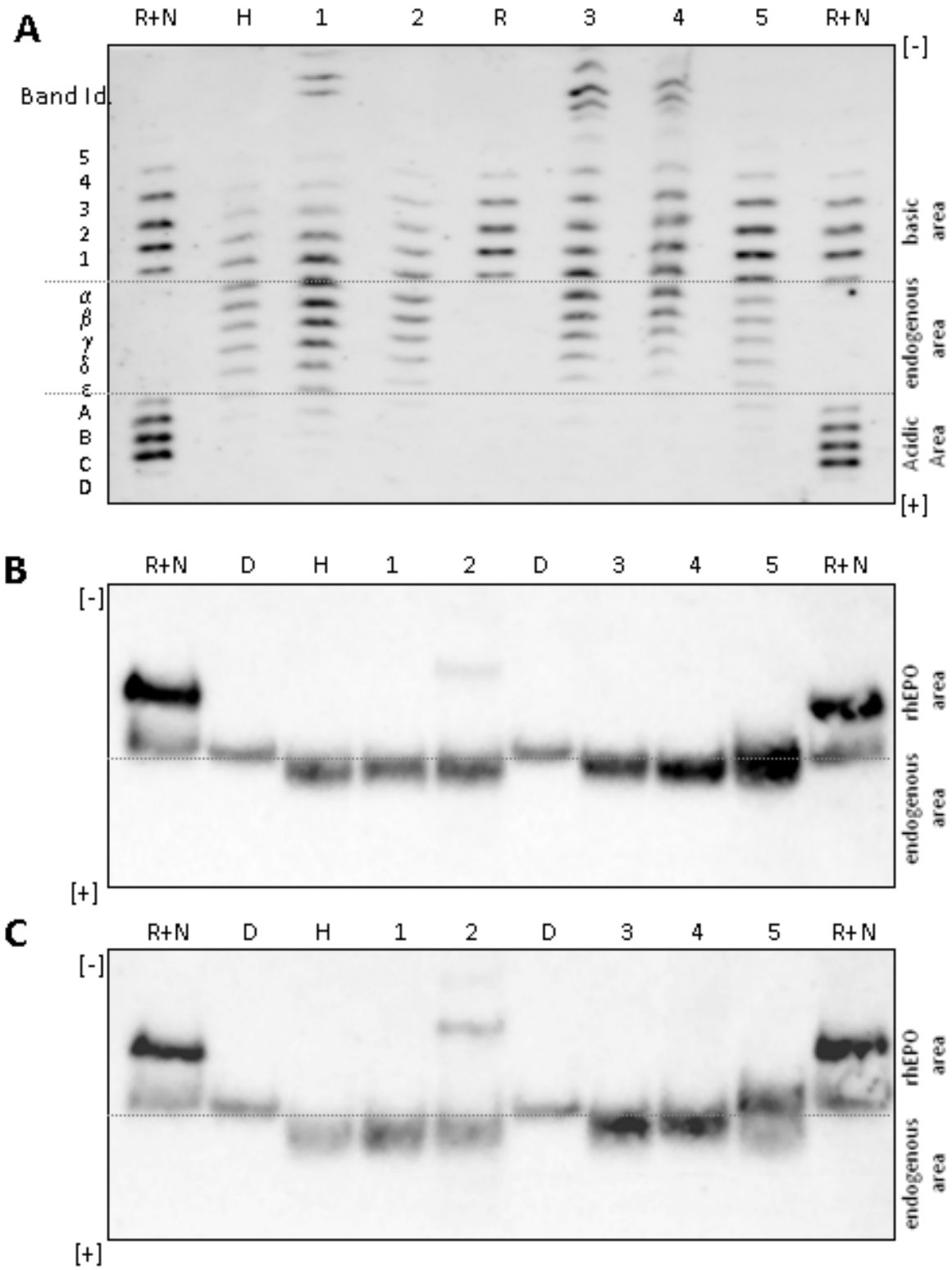

Figure 4: Analysis of EPO in urine and serum samples by IEF and SDS-PAGE with and without neuraminidase treatment: normal profile (lane 1), immunopurified serum (lane 2), atypical "effort-like" athletes' urines (lanes 3 and 4) and rhEPO: huEPO 2: 1 mixture (lane 5) analysed by A) IEF; B) SDS-PAGE and C) SDS-PAGE with neuraminidase treatment. (R: epoetin-α/β; N: darbepoetin; H: huEPO and D: epoetin-δ).

ISBN 978-3-86884-038-4

References

1) Lasne F, de Ceaurriz J. (2000) Recombinant erythropoietin in urine. *Nature* **405**, 635.
2) Desharnais P, Ayotte C. (2007) Towards the screening of urinary EPO by SDS-PAGE analysis. In: Schanzer et al. (eds) Recent advances in doping analysis (15). Sportverlag Strauss, Cologne, p. 303.
3) Kohler M, Ayotte C, Desharnais P, Flenker U, Lüdke S, Thevis M, Völker-Schänzer E, Schänzer W. (2007) Discrimination of recombinant and endogenous urinary erythropoietin by calculating relative mobility values from SDS gels. *Int J Sports Med* **29**, 1.
4) Reichel C, Kulovics R, Jordan V, Watzinger M and Geisendorfer T (2009) SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control. *Drug Test Anal* **1**, 43.
5) Kung CK, Goldwasser E. (1997) A probable conformation difference between recombinant and urinary erythropoietins. *Proteins* **28**, 94.
6) Groleau PE, Desharnais P, Côté L, Ayotte C. Low LC-MS/MS detection of glycopeptides released from pmol levels of recombinant erythropoietin using nanoflow HPLC-chip electrospray ionization. (2008) *J Mass Spectrom*. **43**, 924.
7) Lasne F, Martin L, Martin JA, De Ceaurriz J. (2007) Isoelectric profiles of human erythropoietin are different in serum and urine. *Int J Biol Macromol* **41**, 354.
8) Sasaki H, Bothner B, Dell A, Fukuda M. Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. *J Biol Chem*. **1987**; 262: 12059.
9) Takeuchi M, Takasaki S, Miyazaki H, Kato T, Hoshi S, Kochibe N, Kobata A. Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant chinese hamster ovary cells. *J Biol Chem*. **1988**, 263, 3657.
10) World Anti-doping Agency. WADA Technical Document TD2009EPO. Harmonization of the method for the identification of recombinant erythropoietins (i.e. epoetins) and analogues (e.g. darbepoetin and methoxypolyethylene glycol-epoetin beta). http://www.wada-ama.org/Documents/World_Anti-Doping_Program/ WADP-IS-Laboratories/WADA_TD2009EPO_EN.pdf. Accessed July 22, 2012.

Acknowledgements

The skilful assistance of Claudine Hamelin and Charles Stinfil is gratefully acknowledged. This work was done with the support of the Canadian Centre for Ethics in Sport.