In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (14). Sport und Buch Strauß - Köln 2006

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Intermediate neuraminidase activity: a possible link toward suspicious or "effort-like" EPO isoelectric profiles

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INTRODUCTION

The results of the test currently employed to detect urinary erythropoietin (EPO) are not always conclusive, especially when faced with profiles containing a high percentage of basic isoforms. This is the case for "active urines" samples in which some enzymatic activity is responsible for the EPO pattern disappearance or its shift towards basic values of the pH range[1], and for what is referred to "effort-like profiles", that are atypical profiles of natural EPO generated following short and strenuous exercise[2]. The "effort" profiles are characterised by a high percentage of basic isoforms making up for 50 to 70% of the total; such atypical profiles can be adequately differentiated from recombinant EPO following established positivity criterias.

Here, in a case report format, we wish to present and discuss profiles composed predominantly of bands co-localised with rhEPO basic isoforms. We selected four EPO profiles of athlete's urine samples received and analysed in our laboratory, in which 67 to 73 % of the isoforms present were localised in the basic region. Such profiles appear at first sight as "effort-induced" or suspicious, since almost all criteria for reporting adverse findings were met (e.g. sample D). Although they were not typical of active urines, stability tests were performed on these samples. Interestingly, for all of them we noted the presence of a shifting activity, therefore questioning the origin of these profiles.

One explanation could be that these profiles correspond to the intermediate stage of an enzymatic activity on urinary EPO, having altered the original EPO urinary profiles of these samples. Unfortunately, without performing the stability test, examination of the profiles reveals no sign of enzymatic activity. Although this activity does not generate deviant profiles

that should be interpreted and reported as adverse findings, it shows that utmost care should be exercised when faced with such profiles.

RESULTS AND EXPERIMENTAL

The profiles we wish to report here were obtained from routine samples received in the laboratory for analysis. Of a total of 15 samples selected for their high basic percentage IEF profiles and submitted to stability tests, 6 demonstrated the presence of a high (sample B and D), average (sample A and C) or low (not presented) shifting activity. **Figure 1** shows the IEF profiles from screening analysis (as described by F. Lasne et al. 2000)[3], and the stability tests following the WADA technical document, TD2005EPO[4]. **Stability tests**: 600 µl of urine was concentrated on Microcon YM-30 and the volume was adjusted to 600 µl with a solution of 50mM sodium acetate pH 5.0. rEPO (30 mU/20 µl) and NESP (0.5 ng/20 µl) were added to the sample and incubated overnight at 37°C. 20 µl were analysed by IEF.

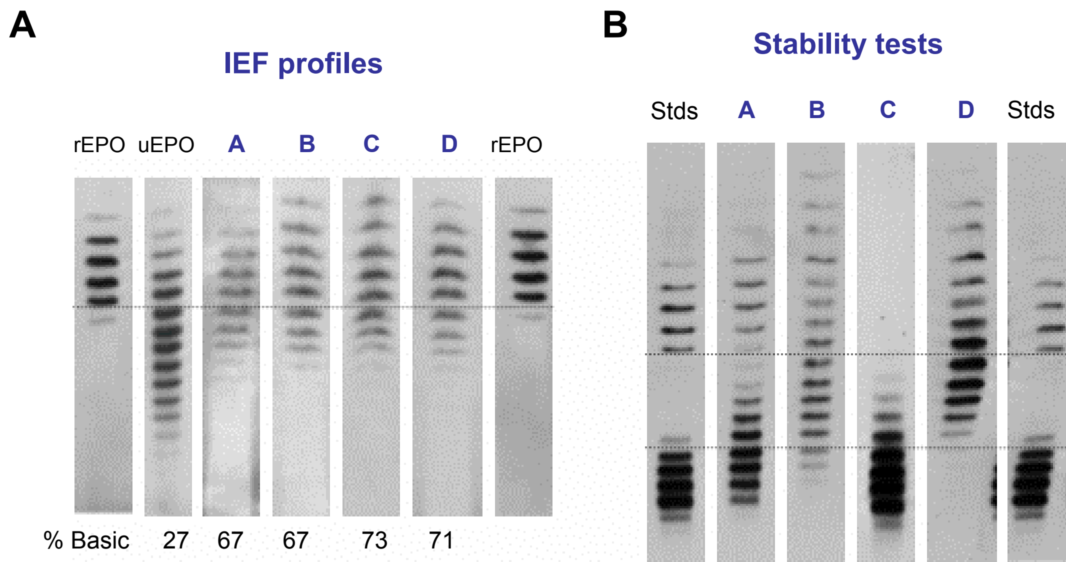

Figure 1 : A) IEF profiles of 4 samples (A, B, C, D) obtained following screening analysis **B)** Results of the corresponding stability tests – all revealing the presence of an inherent activity.

Generation of profiles with « elevated » percentage of basic isoforms from active urines: effect of temperature: Incubation of reference standard uEPO (NIBSC) with «active» urine samples concentrated four times. **Figure 2 A, B, C**: Sample D 4X was incubated with 50mU uEPO/lane for periods ranging from 4 hours to 168 hours (7 days). As shown in figure 2C lane 96+, enzymatic activity is still present in the specimen already exposed to an incubation

of 96 hours to which uEPO was added and incubated again for further 72 hours. Three other shifted profiles, obtained from sample A, B or C incubations with reference standard uEPO, are also presented in figure 2D.

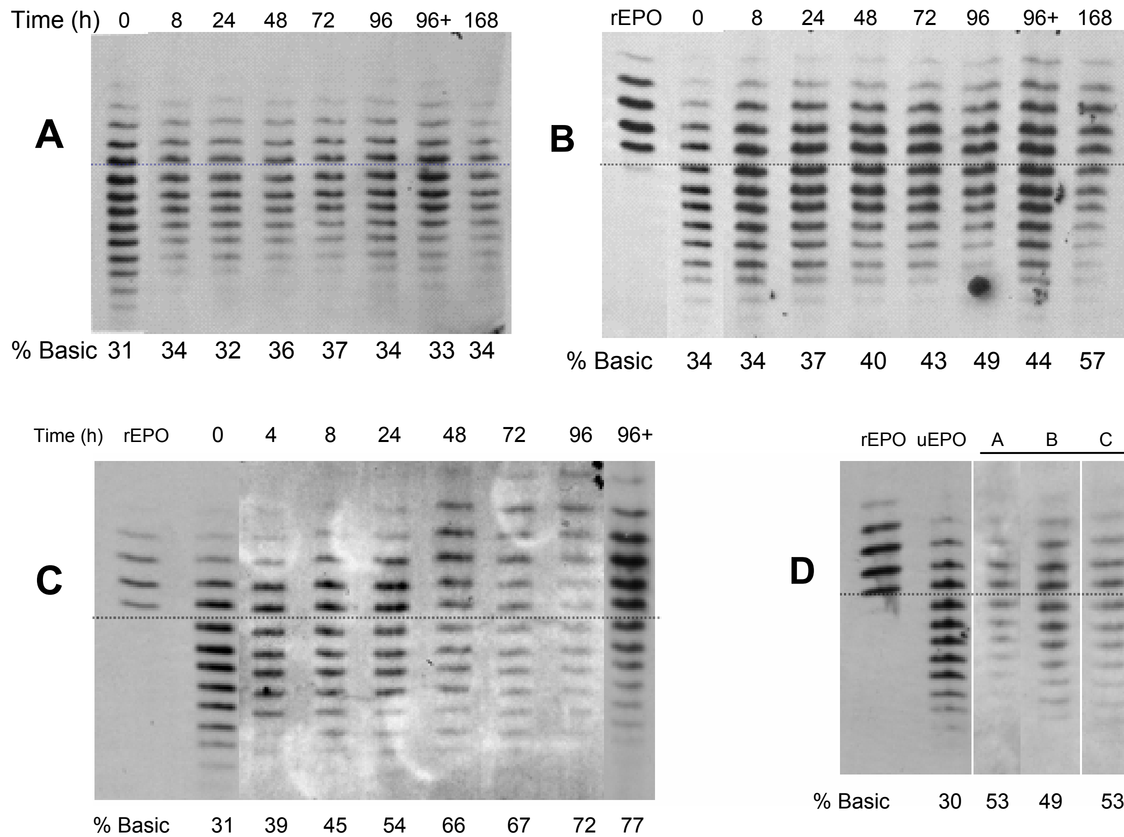

Figure 2: Results of sample D 4X incubations carried out at 4ºC **(Panel A),** room temperature **(Panel B),** and 37 ºC **(Panel C)**. **Panel D)** Other shifted uEPO profiles obtained from the incubations with samples A, B and C.

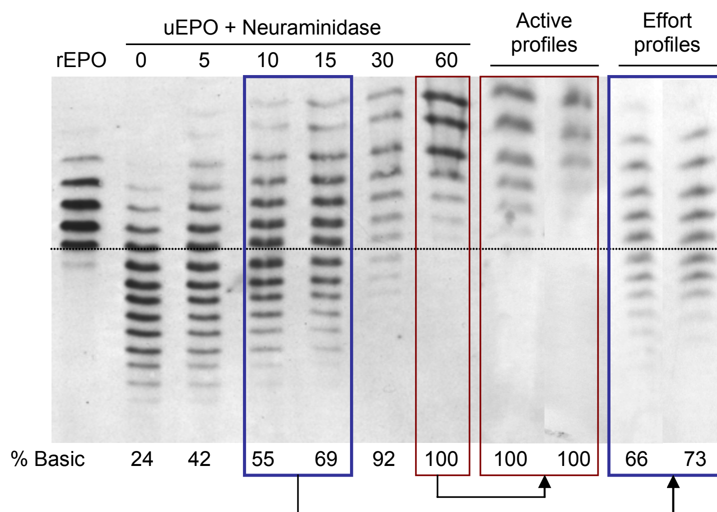

Figure 3: Neuraminidase activity on uEPO developing with time; profiles obtained are compared to active and "effort-like".

We observed that atypical/"effort-like" and active urine profiles were generated by the activity of neuraminidase on uEPO at mid-stage and end-stage respectively. uEPO (50 mU/lane) was incubated with neuraminidase (5 mU) for periods of 5 to 60 minutes at 37°C in sodium phosphate 20mM, at pH 7.4. Results are presented in **figure 3**; profiles were compared to typical effort of active urine profiles.

Finally, it is interesting to stress again that the efficacy of the EPO test is improved when the urine samples are sent rapidly to the laboratory following the collection. In 2005, EPO isoforms were not detected in 15.3% of the samples submitted to analysis in our laboratory (n = 800) and 3.3% were "active urine". By comparison, the samples collected during the 2005 FINA Championships (n = 105) were immediately delivered to the laboratory (within 1 to 4 hours after collection) and frozen until analysis; in such conditions, the number of undetectable EPO and active urine findings were both significantly decreased to 1.8%.

CONCLUSIONS

The classification of EPO profiles as originating from "active" urines should not be restricted to the end-stage profiles typically expected. We have observed and reported that enzymatic activity may be present in samples not initially suspected of being active. The intrinsic activity in these samples was shown to modify and shift the profile of reference standard uEPO isoforms in the basic region increasing its basic isoforms percentage. The neuraminidase activity on reference uEPO can generate intermediate and end-stage profiles similar to "effort-like" or active urines, which seems to indicate an implication of that enzyme in the appearance of "effort-like" or active urine profiles. The origin of the phenomenon leading to intermediate or end-stage profiles is not fully understood and need to be further studied.

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[1] F. Lasne, L. Martin and J. de Ceaurriz, Active urine and detection of recombinant erythropoietin, Recent Advances in Doping Analysis (13). Sport und Buch Strauss, Köln 2005, 297-304.
[2] F. Lasne, Blood, 108 (5), 1779 (2006).
[3] F. Lasne and J. de Ceaurriz, Recombinant erythropoietin. 2000. Nature. 405(6787), 635.
[4] WADA Technical Document TD2004EPO, Harmonisation of the method for the identification of epoetin alfa and beta (EPO) and Darbepoetin alfa (NESP) by IEF-Double blotting and chemiluminescent detection, at www.wada-ama.org.