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Identification of mixed profiles in EPO routine analysis

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Abstract

From 2010 to 2012 the cologne laboratory has screened an increasing number of routine urine samples with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for rEPO. The number of rEPO suspicious samples has increased coinstantaneously. Disturbingly, some of the results obtained through isoelectric focussing polyacrylamide gel electrophoresis (IEF-PAGE) confirmation procedure showed inconspicuous isoform profiles. Often the results did not meet the criteria for an *Adverse Analytical Finding* (AAF) as defined by the World Anti-Doping Agency (WADA) technical documents (TD2009EPO, TD2013EPO). In routine analysis, SDS-PAGE has shown to be superior to the IEF-PAGE when detecting mixed profiles containing both EPO of endogenous (uEPO) and recombinant origin (rEPO). With SDS-PAGE trace amounts of rEPO were detectable despite abundant amounts of uEPO were present in the samples. This was not possible when applying the IEF-PAGE. The data presented show that reliable identification of these mixed profiles relies on SDS-PAGE followed by low-background immunoblotting.

Introduction

Erythropoietin (EPO) is a peptide hormone responsible for hypoxia-induced promotion of erythrocyte production [1]. Its capability to enhance oxygen transport capacity, mask suspicious blood values or reduce regeneration time after withdrawal of blood leaves EPO with a high potential for misuse in sports [2,3]. A direct test for EPO abuse was introduced in 2000 using IEF-PAGE to distinguish uEPO isoform profiles from recombinant ones [4]. In 2007 a second method was implemented, the SDS-PAGE [5,6]. This method separates uEPO from rEPO by molecular weight. The criteria which both procedures need to fulfil for the identification of rEPO in a doping control sample are defined by WADA (TD2009EPO, TD2013EPO). A sample containing both rEPO and endogenous EPO gives rise to mixed profile. These mixed profiles are challenging to identify and interpret correctly.

Experimental

From 2010 to 2012 a total of 10,400 urines samples from anti-doping control were tested for rEPO by the doping control lab in Cologne. 3500 were analyzed using IEF-PAGE while 6900 were analyzed using SDS-PAGE. Samples were prepared via ultrafiltration [4] followed by an either IEF-or SDS-PAGE specific immunoaffinity purification using ELISA-wells (Stemcell; Vancouver, Canada) [5,6,9]. Samples were then separated with either IEF-PAGE [4] or SDS-PAGE [3,4] followed by immunoblotting and detection via chemilumiescence.

Results and Discussion

In the years from 2010 to 2012 about one third of all samples were screened with IEF-PAGE and two thirds were tested with SDS-PAGE. This was done to gain practical experience with both methods assuming that the methods are comparable in sensitivity. In 2010 and 2011 eight AAF were reported and eight samples were found suspicious. In order to report a suspicious sample as an AAF the identification criteria of the TD have to be met. For IEF-PAGE the TD in force at the time

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(TD2009EPO)[7] required three consecutive EPO isoform bands in the defined basic area. The bands defined as “1” and “2” or the bands “2” and “3” had to be about twice as intense as any band in the defined endogenous area or about 85% of the overall intensity of the bands measured by densitometry had to be in the basic area. Figure 1 shows SDS-PAGE (1A) and IEF-PAGE (1B) analyses of a sample, which was found suspicious in 2010 but did not fulfil the identification criteria for IEF-PAGE of TD2009EPO as explained above. SDS-PAGE analysis revealed a faint rEPO signal above the uEPO band. The sample obviously contained rEPO but could not be reported as an Adverse Analytical Finding due to the identification criteria defined for IEF-PAGE. All eight suspicious samples of 2010 and 2011 showed a faint rEPO signal on SDS-PAGE but did not meet the identification criteria. Intrigued by these results, the initial testing procedure of our clients was altered in 2012. The percentage of urine samples screened with SDS-PAGE was not increased substantially, but urine samples from sport disciplines with higher prevalence for EPO abuse (e.g.: cycling, biathlon...) were screened solely with SDS-PAGE. In 2012 five AAF were reported and 19 suspicious samples were found. Not only the increase of suspicious samples was surprising, but also the suspicious samples themselves. Figure 2 shows the SDS-PAGE (2A1, 2A2) and IEF-PAGE (2B1, 2B2) analyses of two suspicious samples which were found in 2012. Both samples showed a faint signal of rEPO above the uEPO band on SDS-PAGE. Sample 1 could be classified as suspicious with IEF-PAGE analysis (B1) but did not fulfil the criteria of TD2009EPO. Sample 2 was unsuspicious in the IEF-PAGE analysis. The sample was screened with SDS-PAGE and would surely not have been found if screened with IEF-PAGE alone. In march 2013 TD2013EPO came into force with mitigated IEF-PAGE identification criteria for rEPO. While the TD2009EPO required twice the intensity of the bands designated “1” and “2” or “2” and “3” of any endogenous band, the new document only requires the two most intense bands to be in the basic area [8]. These criteria will lead to reporting of more suspicious samples as AAFs. But samples as the ones shown in Fig. 2 will still be missed. Both samples contain rEPO but the criteria of TD2013EPO for IEF-PAGE though mitigated can still not be fulfilled.

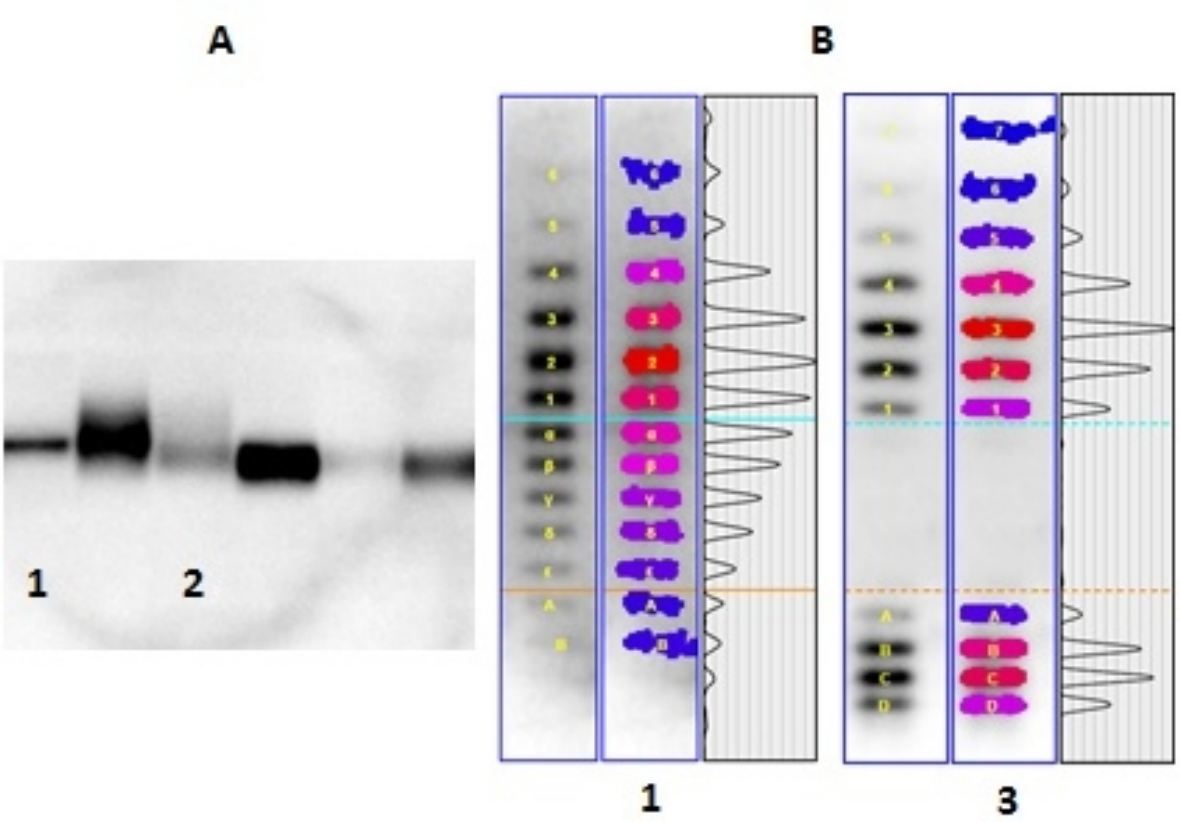

Fig 1: Immunoblots of an SDS-PAGE (A) and IEF-PAGE (B)analysis of a urine sample and rEPO standard. Lanes of the Dynepo standard (1), the sample (2) and BRP/NESP standard (3) are marked.

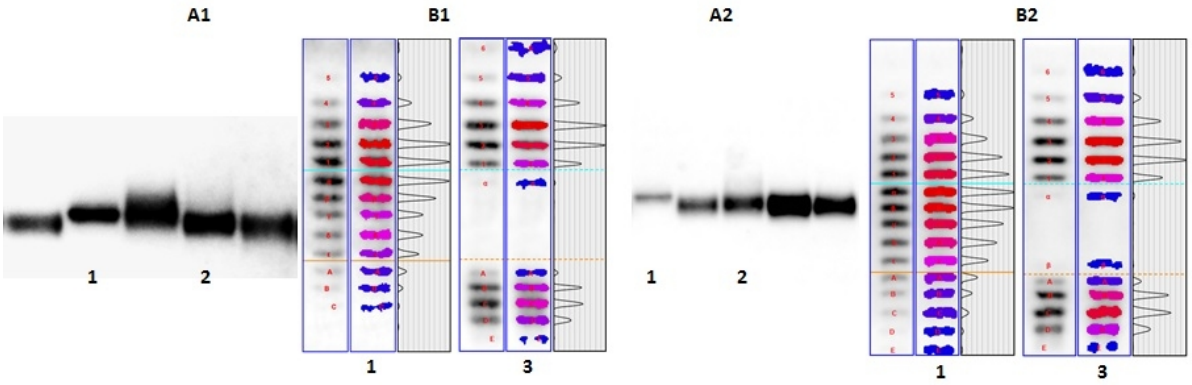

Fig 2: Immunoblots of an SDS-PAGE (A1, A2) and IEF-PAGE (B1, B2) analysis. Lanes of the Dynepo standards (1), the samples (2) and BRP/NESP standards (3) are marked.

Conclusions

In routine analysis SDS-PAGE with its separation by molecular weight has proven to be superior to the separation by isoelectric point. If the screening for rEPO is performed by IEF-PAGE suspicious samples will inevitably be missed. A suspicious sample found by SDS-PAGE screening should also be confirmed using SDS-PAGE. So far this is not possible since the TDs (2009, 2013) require IEF-PAGE analysis for confirmation in case the screening was performed with SDS-PAGE or with the newly established SAR-PAGE. There is need for a revision of the technical document to open the possibility for confirmation of suspicious samples using SDS-/SAR-PAGE only.

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