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Possible origins of undetectable EPO in urine samples

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Abstract

Background: In order to determine the possible origins of undetectable EPO profiles in athletes' urine, we analyzed the data obtained from a large number of official anti-doping urine tests aimed at detecting recombinant erythropoietin. The following variables were considered as potential causes for lack of EPO detection: athlete's gender, competition effect, urine specific gravity as well as possible usage of proteasic adulterants to evade doping detection.
Results: Statistical analyses indicated that undetectable EPO profiles were clearly related to urine properties such as low EPO concentrations or extreme specific gravities. The addition of very small quantities of protease was shown to remove all traces of EPOs in urine. This finding led to the development of a simple, specific and sensitive test that reveals proteasic activity based on albumin digestion.
Conclusions: Urine characteristics clearly affect the detectability of an EPO profile. At the same time, addition of anti-proteases prevents the adulteration of urine. These two findings have clear practical implications with regards to the timing of urine collection as well as the entire anti-doping control procedure.
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1. Introduction

Erythropoietin (EPO) is a native human glycoprotein hormone, which main physiological effect is the induction of erythrocytosis and the consequent improvement of blood oxygen-carrying capacity [1]. Because an increase of the number of erythrocytes enhances athletic performances in endurance sports [2], the use of synthetic forms of EPO is prohibited by the World Anti-Doping Code. Indeed, EPO analogs, such as recombinant human EPO (rHuEPO), can substitute for endogenous EPO by binding to the EPO receptor and triggering intracellular signaling in a manner identical to that of the native hormone.

Since more than a decade, endogenous and recombinant EPOs have been reported to be separable according to their charge heterogeneity[3–4]. Beginning 2000, a method developed by Lasne [5–7] and based on isoelectric focusing (IEF) of EPO in polyacrylamide gels followed by double-blotting was published and validated. Currently, and in spite of occasional controversy surrounding its use [8–10], EPO isoelectric focusing is the only official method used on a routine basis in all World Anti-Doping Agency (WADA) accredited anti-doping laboratories.

At the same time, the evaluation report of the urine EPO test published by Thormann and Peltre [11] and other unofficial data indicate that approximately 15% of all EPO tests carried out in anti-doping laboratories yield undetectable EPO profiles. An EPO profile is considered undetectable if no endogenous or recombinant EPO can be detected in a sample using the classical IEF-based test. In order to determine whether the origin of undetectable EPO profiles could be traced back to specific sample characteristics such as urine specific gravity or athlete gender, we decided to carry out a statistical analysis of the

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results obtained in more than 3000 EPO tests. Some of the samples were also analyzed for their actual EPO content to establish the relation between EPO concentration and EPO detectability.

In the last few years, it has been observed that several athletes systematically present undetectable EPO profiles. According to rumors circulating among top level endurance athletes, the results of an EPO test may be in fact tampered with by the addition of specific adulterants [12]. These rumors were partially substantiated by recent seizures of doping agents, including unidentified, but closely related products, as reported on the official WADA website (http://www.wada-ama.org). Proteases are potential candidate adulterants that may act as masking agents in an EPO test. To verify this assertion, we decided to test whether the addition of a very low quantity of a common protease would be sufficient to eliminate all visible traces of endogenous and exogenous EPO in an IEF-based test. Our experiments were also aimed at demonstrating that the addition of a concentrated mix of protease inhibitors can prevent EPO digestion by different proteases.

Our findings can be used to improve EPO testing in several ways. Firstly, our large statistical analysis enabled to foresee the possible origin of undetectable EPO in urine samples. Secondly, in case of undetectable EPO profiles, we propose the systematic use of a simple, very sensitive and reliable analysis of a urine endogenous protein marker to reveal protease alteration and thus the addition of exogenous protease into the sample. Finally, we believe that the timing of urine collection may be improved and the entire protocol of EPO testing may be refined as to minimize possible degradation of peptides hormones following malevolent addition of exogenous protease.

2. Material and methods

2.1. Samples

3050 negative routine samples were analyzed for EPO in our laboratory between 2003 and 2006 and included in the statistical analysis. Negative samples demonstrated an isoelectric EPO profile identical to the urinary negative standard ("detectable"). Moreover, samples without any profile are considered as negative ("undetectable"). After analysis, they were stored at −20 °C and discarded after 3 months, so that no freeze–thaw cycles are performed. According to the urine collection (in-competition/out-of-competition), the samples were also subjected to the other mandatory anti-doping analyses. Most samples were collected from endurance athletes (athletics, cycling, skiing, etc.).

Of this total of 3050 samples, 92 were collected during the same unique major competition event. Because pre-analytical conditions of these 92 samples, such as collection, storage and time delay, were standardized and optimized, they were chosen for subsequent EPO dosage experiments.

2.2. EPO isoelectric focusing

Isoelectric focusing (IEF) was performed as previously described by Lasne et al. [13] and mandated by WADA for anti-doping EPO tests. Isoelectric profile analysis was performed using "GASepo" v1.2 software from Smart Systems [14].

2.3. Addition of proteases to detectable samples

A series of 20 ml aliquots of a negative detectable urine sample were spiked with various concentrations of trypsin (trypsin from porcine pancreas, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (0 μg/ml, 0.5 μg/ml, 5 μg/ml and 50 μg/ml, respectively). Immediately prior to the addition of 50 μg/ml of trypsin, one of the samples was spiked with protease inhibitors to a final concentration of 1 μM, using a concentrated solution of protease inhibitors, containing 2 ml of Complete (Complete™ Protease Inhibitor Cocktail, Roche Pharma AG, Reinach, Switzerland) and 2 ml of pepstatin (pepstatin A synthetic, Calbiochem, San Diego, USA). All urine aliquots were incubated overnight at room temperature with gentle shaking. Retentates were prepared from these aliquots and electrophoresed in an IEF gel for EPO analysis. Alternatively, 1 ml of urine was precipitated prior to Western blotting for albumin analysis (seeSection 2.5).

The same was done for the preparation of urine samples degraded with protease from Streptomyces griseus type XIV (Sigma-Aldrich Chemie GMBH, Steinheim, Germany), pepsine (Sigma-Aldrich Chemie GMBH, Steinheim, Germany), papain (Sigma-Aldrich Chemie GMBH, Steinheim, Germany) and proteinase K (Sococchim SA, Lausanne, Switzerland).

2.4. Total protein and total EPO quantification

Total protein content in urine samples was measured using a pyrogallol red assay (Autokit Micro TP, Wako Chemicals GmbH, Neuss, Germany).

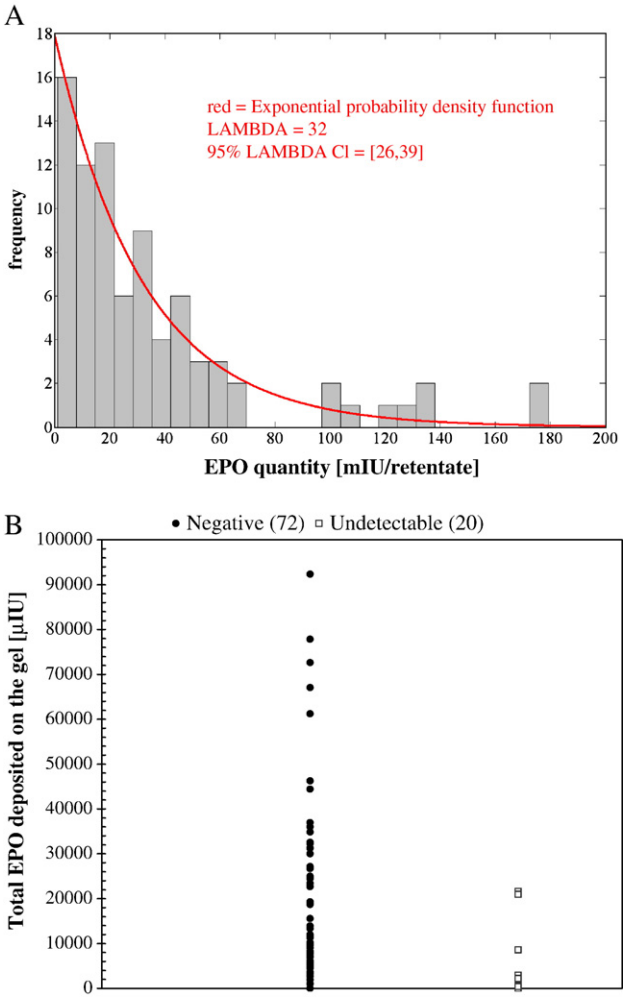

Fig. 1. A. Distribution of the EPO amounts [mIU/retentate] extracted from 20 ml of 92 routine urine samples. The distribution frequency curve fits a log–normal curve. B. Classification (detectable/undetectable) of 92 negative urine samples, following the classical used EPO analysis method described by Lasne [3–5], according to the total amount of EPO deposited on the gel. Note that all samples with the highest EPO concentrations (more than 25,000 μIU EPO deposited on the gel) were detectable. In contrast, all undetectable samples (N=20) had lower EPO concentrations, even if some samples with very low EPO concentrations were also detectable.

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