

## Detection window of Darbepoetin- $\alpha$ following one single subcutaneous injection

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### Abstract

**Background:** The current official direct recombinant erythropoietin (rHuEPO) detection anti-doping test based on 1D isoelectric focusing (IEF) of urinary proteins was performed to determine the detection window of Darbepoetin- $\alpha$  when applying the positivity criteria established by the World Anti-Doping Agency (WADA).

**Results:** Following WADA's positivity criteria, the IEF based urinary EPO test enabled to determine that the detection window after a single subcutaneous injection of Darbepoetin- $\alpha$  (40  $\mu$ g of ARANESP<sup>®</sup> injected) is close to 7 days, that is to say approximately two times more than for rHuEPO- $\beta$  (4000 IU of Recormon<sup>®</sup> injected). The detection window can be different from one subject to another, because the actual positivity criteria take into consideration in some way the endogenous EPO production rate which differs enormously from one subject to another. That means, all subjects with a naturally elevated or stimulated EPO production rate (altitude training, hypoxic tent,...) have a reduced detection window for bone marrow stimulators such as Darbepoetin- $\alpha$ .

**Conclusion:** Darbepoetin- $\alpha$  has a much longer detection window in urine than any other available EPOs, which is a major disadvantage for illegal use in sports. The positivity criteria used up to now by all anti-doping laboratories are very conservative. Furthermore all athletes tested for rHuEPO are not equal regarding the actual test. For that reason, the criteria could be slightly adapted in the future, but further experiments are needed.

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**Keywords:** Erythropoietin; NESP; Detection window; Doping; Urine

### 1. Introduction

Novel Erythropoiesis Stimulating Protein (NESP, Darbepoetin- $\alpha$ ) is a 38.5 kDa glycoprotein hormone that differs from native human erythropoietin (EPO) by five amino acids, which allow the attachment of additional oligosaccharide chains [1]. NESP isoforms contain up to 22 sialic acid residues compared with a maximum of 14 sialic acid residues for native EPO isoforms. NESP can substitute for endogenous EPO by binding to the EPO receptor and inducing intracellular signalling in an identical manner as the native molecule. It is known that sialic acid residues on EPO are responsible for maintaining its biological activity *in vivo* [2]; indeed, NESP has been shown to

have an increased half-life as well as an increased biological activity *in vivo* when compared with human native EPO [3].

The main physiological effect of NESP is the induction of erythrocytosis and consequently the improvement of oxygen-carrying capacity of blood. NESP has been reported to be used as a doping agent by three athletes performing in the 2002 Winter Olympic Games in Salt Lake City. However, as NESP has been shown in blood to have a significantly longer half-life than other EPO analogues, such as recombinant human EPO (rHuEPO) [4], its longer detection window appears to be a disadvantage in case of illegal use in sports.

As originally described by Lasne [5], EPO molecules isoforms can be highlighted by isoelectrofocalisation on a polyacrylamide gel followed by a double blotting [6]. The number of charged molecules, such as sialic acid residues, influences the isoelectric point and consequently the position of the isoforms on the gel. Therefore, NESP isoforms can be differentiated from native EPO

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Table 1  
Characteristics of the subjects and haematological parameters

Subject	Age	Weight [kg]	Height [cm]	Day 0		Day 14		Day 18		Day 22		Day 25	
				Hb[g/l]	Ret[%]	Hb[g/l]	Ret[%]	Hb[g/l]	Ret[%]	Hb[g/l]	Ret[%]	Hb[g/l]	Ret[%]
S1	22	72	180	145	1.0	138	1.8	158	1.5	152	0.8	155	0.8
S2	24	66	172	143	1.2	162	1.8	160	1.9	153	0.8	155	0.6
S3	23	78	188	150	0.9	157	1.6	154	2.0	155	0.9	158	0.7

\* Reticulocyte count increase is significant between Day 0 and Day 18 ( $P < 0.05$ ).

isoforms as the bands corresponding to the NESP isoforms can be detected in a much more acidic area than that of the endogenous EPO. At the moment, this method is the only one officially used on a routine basis, even though it has been occasionally subject to controversial [7,8].

In this study, we aimed to investigate the excretion kinetics of NESP in urine after a single injection of 40 µg ARANESP®. WADA's positivity criteria were evaluated and applied to determine the detection window of NESP. An alternative representation model was also proposed to facilitate the interpretation of an isoelectric profile indicating the presence or absence of NESP in urine.

## 2. Material and methods

### 2.1. Subjects characteristics

Three healthy Caucasian men between 22 and 24 years of age participated in the study (see Table 1), which was conducted according to the Declaration of Helsinki as amended in the 41st World Medical Assembly (Hong Kong 1989) and was approved by the local ethical committee. All subjects did not practice semi-professional or professional sport and had to go through a complete clinical examination before being included in the study. All participants gave their informed consent and agreed to blood tests and urine collection.

### 2.2. Protocol

All subjects received one single injection of 40 µg (4760 IU) NESP (ARANESP®, Amgen AG, Zug, Switzerland). One urine was collected before injection (T0) and all urines were collected during the week following the injection. Then the first urines of the day were collected during an additional week. Total urine volumes were recorded before the samples were aliquoted and frozen at 80 °C. For safety reasons, blood tests were carried out every three days during the study to control haemoglobin concentration and avoid an excessive blood viscosity increase. Moreover, reticulocyte cell population was quantified with an automatic analyser (Cell-Dyn 3500, Abbott Diagnostics Division, Baar, Switzerland) to control that all subjects responded to the ARANESP® injection.

### 2.3. Total protein and total EPO quantification

Total protein content was measured in urine samples, as well as in the retentates (urine extracts), using a pyrogallol red assay (Autokit Micro TP, Wako Chemicals GmbH, Neuss, Deutschland).

Total EPO content in the retentates was determined using an enzyme linked immunoabsorbant assay (ELISA) (Quantikine IVD Epo ELISA, R&D Systems, Inc., Minneapolis, USA) that is designed to target both endogenous and recombinant EPO isoforms, NESP included [9].

### 2.4. Epo isoelectrofocalisation

IEF was performed as previously described by Lasne et al. [5]. This method was previously used to detect rHuEPO [10] as well as Darbepoetin-α [11], so that the respective isoelectric focusing profiles of non-endogenous EPO are well

established. Briefly, 20 ml of urine was subject to ultrafiltration and then retentates were focused on an IEF gel (pH 2–6). Proteins were double-blotted as described by Lasne [6] and visualized by chemiluminescence (ECL-plus, GE Healthcare, UK) using a supersensitive high-resolution Camera System for chemiluminescence samples (epoCAM, ARC Seibersdorf research GmbH). Isoelectric profile analysis was performed using "GASepo" v1.2 software from Smart Systems [12].

### 2.5. Graphic representation of the results

After EPO isoforms having run from the cathode to the anode, bands were numbered following their position on the obtained isoelectric profile. Band 1 was defined as the second less basic isoform of the standard rHuEPO and the bands towards the cathode were numbered from 2 to 7. Band A was defined as the second less acidic isoform of the NESP standard. The following bands closer to the anode were defined as B to F. The endogenous bands were identified as α to γ from the most basic to the most acidic band. The intensity of each band corresponded to the percentage of the total chemiluminescence signal. Each lane could then be represented by curves defined on a graph with the band number on the x-axis and the percentage of total signal on the y-axis (see Fig. 2).

### 2.6. WADA positivity criteria

To determine if a sample was considered as positive or negative, the WADA positivity criteria were applied (WADA Technical Document — TD2004EPO, October 15, 2004). The following identification criteria defined the requisites that the image must fulfill to consider that an adverse analytical finding corresponding to the presence of NESP has occurred:

- in the acidic area there must be 3 acceptable, consecutive bands assigned as B, C and D in the corresponding reference preparation.
- the most intense bands either measured by densitometry or assessed visually must be C or D.
- the most intense band (C or D) must be more intense than any other band in the endogenous area either measured by densitometry or assessed visually.

Concerning the last criteria, it is generally considered that a signal is unequivocally more intense than another signal if this signal's intensity is

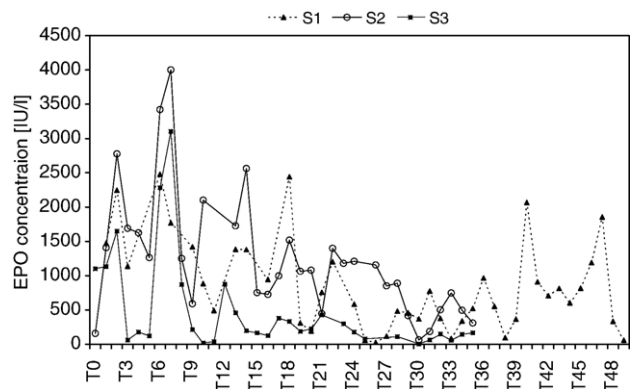


Fig. 1. ELISA measurements of total EPO concentration [IU/l] in all retentates of each subject.

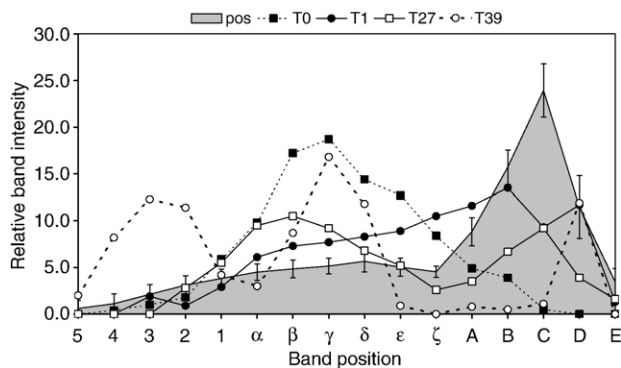


Fig. 2. Bands intensity, expressed in percentage, of T0 and T1 samples (negative), a typical positive profile calculated from the mean of all positive samples (pos), and T27 and T39 samples (suspect) relatively to their position on the gel (Subject 1). Bars represent 95% confidence interval based on standard error of the mean. (+) characterizes the acidic side of the gel (anode). (-) characterizes the basic side of the gel (cathode).

visually at least twice more intense than the other. Urine samples were considered as suspect if one or two of the positivity criteria were fulfilled. Undetectable urine samples were considered as negative.

### 3. Results

Continuous control of blood parameters allowed to attest that all subjects responded to NESP, because of a sudden significant ( $P < 0.05$ ) abnormal increase of the reticulocyte count following the NESP injection (see Table 1). Overall 35 to 49 urines were collected for each subject. Based on the isoelectric profiles, the World Anti-Doping Agency (WADA) positivity criteria were applied for each urine sample.

The first urine sample after NESP injection was either positive or negative, depending on the subject. All the following urines were positive until the seventh day, except for one subject who had one negative urine on the fifth day following the

injection. One subject had, on some occasions, completely undetectable urine samples. Each subject had one urine on the seventh or eighth day following the injection that was negative again. From the ninth day, all detectable urines were negative for each subject. Therefore, according to the WADA positivity criteria, we observed a detection window close to 7 days after a single injection of 40  $\mu\text{g}$  of NESP.

EPO and total urinary protein concentrations were measured in each sample. No correlation could be established between total urinary protein concentration and EPO concentration. For each subject, EPO concentration varied greatly, even when corrected with the specific gravity of the urines, the total volume or the total protein concentration. Except a significant total EPO concentration increase following the NESP injection ( $P < 0.05$ ), no correlation was observed between the EPO concentration and the positivity of the sample. Fig. 1 depicts the ELISA measurements of total EPO concentration in all retentates of each subject.

For a better understanding, some results of one subject, namely Subject 1 (S1), were represented in details in Fig. 2. The maximum peak of a negative urine (T0) was clearly in the endogenous field of the gel. The following urines, including T1, showed a maximum peak in the acidic side of the gel (anode). However, the highest peak of T1 urine corresponded to band B. Therefore, this urine should not be considered as positive according to WADA's positivity criteria. A typical positive profile, calculated from the mean of all positive urines, is also depicted. It can be characterized by its highest peak corresponding to band C. Moreover, two particular urines from the fifth and the seventh day, namely T27 and T39, are shown, as they should not be considered as positive. Indeed, the maximum peak of the positive urines following NESP injection (T2 to T26 and T28 to T38) was most frequently band C and sometimes band D. However, T27 urine's most intense band corresponded to band  $\beta$  in the endogenous portion of the gel; this urine analysis was repeated by three different operators to

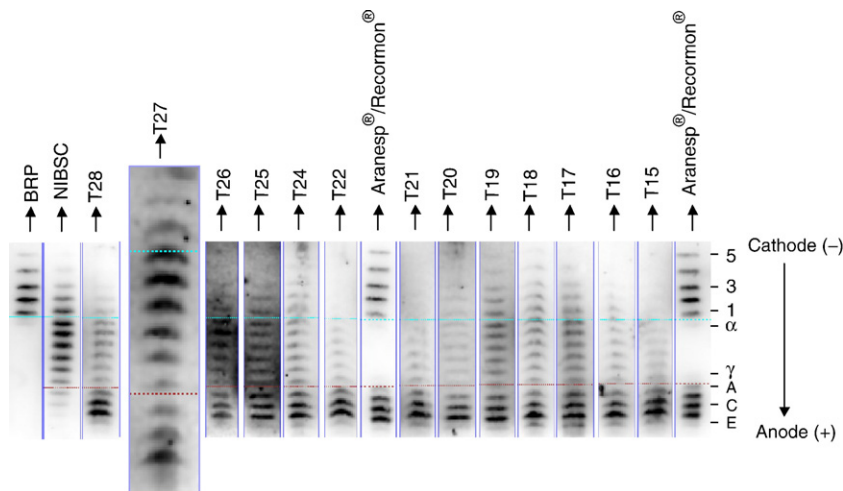


Fig. 3. Gel of T15 to T28 samples (Subject 1) with usual positive controls (Aranesp®/Recormon® standards, BRP (Biological Reference Preparation) standard) and negative control (NIBSC, (National Institute for Biological Standards and Control) standard). Band 1 was defined as the second less basic isoform of the standard EPO- $\beta$  and the bands towards the cathode were numbered from 2 to 7. Band A was defined as the second less acidic isoform of the NESP standard. The following bands closer to the anode were defined as B to F. The endogenous bands were identified as  $\alpha$  to  $\zeta$  from the most basic to the most acidic band. T27 suspect urine sample is highlighted. All other shown samples are positive.

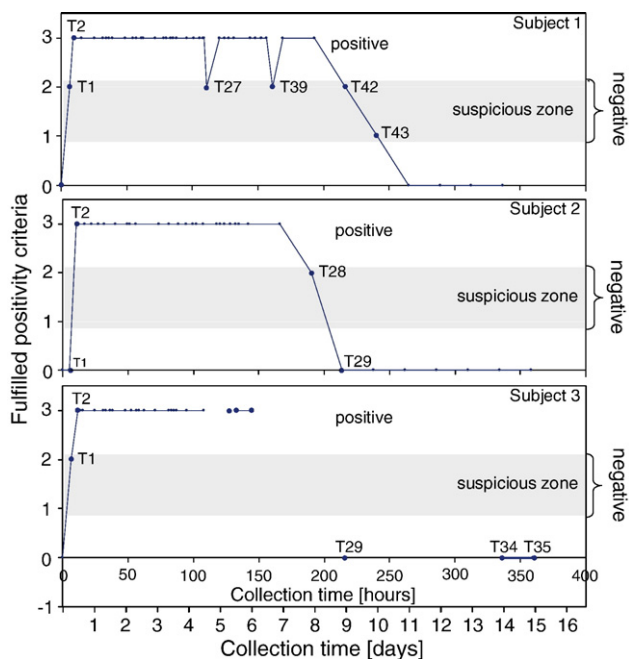


Fig. 4. Number of positivity criteria fulfilled by each urine sample of Subject 1, 2 and 3. Samples within the grey area can be considered as suspect. Undetectable samples were represented by an empty space on the graph. This allows to highlight NESP detection window.

confirm that fact. Thus, this urine was considered as negative. According to the WADA positivity criteria, T28 to T38 urines were positive again. T39 urine, corresponding to the second urine of the seventh day after injection, appeared to be negative for the same reason as T27, even if T40 and T41 urines, corresponding to the seventh and the eighth day following the injection, were still positive. From the ninth day onwards, all urines were negative.

Fig. 3 represents the gel corresponding to the samples T15 to T28. For two subjects, endogenous bands became less intensive following NESP's injection. They almost disappeared for one subject (S3), who had also some completely undetectable samples during the excretion study.

Fig. 4 represents, over time, the number of positivity criteria fulfilled by the samples of all three subjects. According to WADA's rules, all three positivity criteria must be totally fulfilled to declare a sample positive. Samples within the grey area were considered as suspect, but could not be considered as positive according to the WADA rules. Undetectable samples were represented by an empty space on the graph (see Subject 3).

#### 4. Discussion

Following a single 4760 IU NESP injection (approximately 70 IU/kg), a detection window of a minimum of 7 days was observed, according to the WADA positivity criteria. This detection window is considerably longer than for rHuEPO. These results are in accordance with previous publications [13,14]. Indeed, in urine, a single 4000 IU rHuEPO (approximately 70 IU/kg) dose injection shows a detection window of 3 days (unpublished internal data). Therefore, even if NESP's

biological activity is higher than rHuEPO's [3], it can be supposed that in case of same dosage and way of administration, NESP is probably a less adapted doping agent than rHuEPO because of its longer detection window.

Measurements in urine of the total EPO concentrations are usually recommended to standardize as much as possible the test, that is to say the amount of EPO deposited in each lane. However, total urine EPO quantification does not enable to determine the detection window of NESP, and above all the positivity of the sample. The main reasons are that it often happens that samples are close to the limit of quantification, and moreover, the excreted volumes of urine can vary greatly – and consequently the urinary EPO concentration – from one collection to another.

The graphical representation of the results (see Fig. 2) was first published in the Proceedings of the 20th Cologne Workshop on Dope Analysis [15]. The graphs highlight the individual band percentage according to the band position. It is an easy to understand IEF profile representation alternative. For most of the samples, position of the maximum peak constitutes a first positivity indication, as negative samples do not have their maximum peak in the most acidic area of the gel.

During the positivity period, NESP's bands were more intensive than any endogenous EPO bands. For two subjects (S2 and S3), endogenous bands became less intensive following NESP injection. They even completely disappeared on some occasions for one of these subjects (S3). This could be explained by a possible feedback regulation of endogenous EPO production by these 2 subjects [16]. However, published results go against the existing theory which holds that endogenous EPO production is suppressed only when the red cell mass has been increased beyond the homeostatic set point [17]. Moreover, it can happen during an excretion study that some samples are undetectable, even though and according to our experience, NESP should be present in the urine. This probably comes from a low EPO concentration in urine and highlights the limits of the method. It is common practice, in anti-doping laboratories, to return negative results although the profile is undetectable. Unofficial data indicate that, among all anti-doping laboratories, approximately 10 to 15% of all EPO tests show undetectable profiles. In our case, too low EPO concentrations can also explain the observed endogenous bands disappearance in case of a high NESP/endogenous EPO ratio, notably shortly after NESP injection.

During the excretion period, one subject (S1) had two negative samples. Even though the two first WADA positivity criteria were fulfilled for these samples, the last criterium was not. Indeed, the most intense band, when assessed by densitometry, was located in the endogenous field. The same kind of reappearance of endogenous bands was observed in case of rHuEPO micro doses injections [17]. Physiologically, this could eventually be explained by a sudden increase of endogenous EPO production. In our case, as we know in details the whereabouts and the physical activities performed by the subjects, we can exclude a response due to hypoxia following high altitude exposure or strenuous physical activity. However, we know that, among each person, endogenous EPO levels can vary in natural way (up to twofold times) [18,19]. As

suggested by Emslie et al. [20] for rHuEPO, another possible alternative would have been a specific higher endogenous EPO excretion rate compared with NESP. However, no significant pH or EPO concentration difference was noted for this specific sample when compared with other samples of the same subject. As two clearly positive samples should have been considered as negative, the relevance of the last positivity criteria can be subject to discussion. If we consider the endogenous EPO production rate, all athletes are not equal when this specific criterium is applied. Subjects with a natural elevated endogenous EPO production and abusing of NESP will be more often considered as negative as people with slightly lower endogenous EPO production. The same situation exists in case of rHuEPO doping [17]. For that reason, in case of NESP abuse, the main considered information should not be the band intensity ratio, but rather the position and the specific distribution of the bands in the most acidic area of the gel; these latter must be in agreement with those of a positive NESP standard. Consequently, in case of NESP abuse, the last WADA criterium considering intensity ratio could be removed. Indeed, on the contrary to atypical negative urines samples [21] which can potentially be confused with a recent intake of rHuEPO, the risk of declaring a NESP false positive result is extremely improbable. Indeed, no modification of the IEF profile has been observed up to now in the acidic area of a gel following a strenuous physical effort. More generally, after having performed approximately 6000 urinary EPO anti-doping tests, we have never observed any atypical or even doubtful profile in the very acidic and specific domain of NESP in a gel.

For that reason, we can assume that the actual criteria are too conservative.

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