Applications and Biomonitoring Issues of Recombinant Erythropoietins for Doping Control

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Abstract: The biochemical actions and side effects of recombinant erythropoietins (rhEPOs), their analogs and mimetics, their misuse as doping agents, and the principal analytical strategies developed to identify them in athletes' biologic fluids are reviewed. Patients who experience a range of pathologies have benefited from the administration of rhEPOs to correct severe anemia. Currently, monitoring the biologic effect of rhEPO in patients under treatment is by measuring the hemoglobin concentration. However, it may be valuable to directly monitor the actual levels of the administered drug and determine a dose-dependent correlation with any clinical adverse effect observed. This may permit the adoption of a patient-specific administration regime. Currently, the method of detecting EPO approved for doping control is an isoelectric-focusing, double-blotting, chemiluminescence assay based on charge differences between isoforms of rhEPOs and endogenous EPO in urine. The advantages and limitations of this method are presented. A new approach using sodium dodecyl sulfate–polyacrylamide gel electrophoresis as a complementary tool to the established method is discussed. The application of matrix-assisted laser desorption/ionization mass spectrometry and liquid chromatography combined with tandem mass spectrometry for the direct detection of the rhEPO molecules may prove to be promising. Indirect evidence of rhEPO abuse by athletes is based on the analysis of blood parameters (hemoglobin hematocrit, reticulocytes, macrocytes, etc) and serum markers (concentration of EPO and serum transferrin receptors, etc). Enrichment of the screened parameters with gene or biochemical markers revealing altered erythropoiesis and adoption of longitudinal monitoring of athletes' hematologic and biochemical parameters could also be a complementary approach in the fight against doping.

Key Words: recombinant erythropoietins, medical applications, doping

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INTRODUCTION

Human erythropoietin (hEPO) is a glycoprotein hormone synthesized predominantly in the kidneys, which stimulates erythroid proliferation and differentiation. It acts synergistically with other cytokines on bone marrow colony-forming unit-erythroid cells to cause maturation and proliferation of the normoblast stage of eyrthroid cell development.[1–4] The commercial availability of recombinant human EPO (rhEPO) products and EPO analogs and mimetics has facilitated its widespread therapeutic application. The increase in circulating erythrocytes may also be used to increase oxygen delivery to muscle, improving performance in sport.[5] Currently, EPO and any analog or mimetic thereof is also included in the World Anti-Doping Agency's (WADA) List of Prohibited Substances.[6] Although an official method exists for detecting rhEPOs,[7] that is validated by accredited laboratories and accepted by WADA, the efficacy of this method has come under criticism in the last 4 years. Furthermore, current methodologies for direct detection of rhEPOs fail to cover the vast variety of its analogs and mimetics. This review summarizes the existing approaches, comments on analytical methodologies for biomonitoring of rhEPOs, and suggests possible improvements and alternatives.

Structure of Human Erythropoietin and Recombinant Human Erythropoietins

The gene encoding for hEPO is located on chromosome 7 of the human genome and encodes a protein of 193 amino acids (AA), including a 27 AA signal peptide. The intact protein consists of a 165 (DesArg166) or 166 AA sequence containing three N-glycosylation (Asn24, 38, and 83) sites and one O-glycosylation (Ser126) site[8,9] with a calculated molecular weight of approximately 30 kDa and a carbohydrate moiety accounting for 40%.[10–12]

In 1985, Lin et al isolated the hEPO gene from a genomic phage library and were able to characterize it for research and production.[10] The isolation of the hEPO gene enabled the transfection of mammalian cell lines (including Chinese hamster ovary or baby hamster kidney cells) for the

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large-scale production of rhEPO by biotechnologic methods.[13] This resulted in a successful clinical trial of a synthetic form of the hormone, Epogen,[14] and the first rhEPO (epoetin-alpha) was approved in 1987 by the Food and Drug Administration for therapeutic use in the United States. Since then, and especially since December 2003, when the patent for Epogen expired, several rhEPOs, derivatives, analogs, and mimetics have become available as therapeutic agents (Table 1).[15–21]

Several studies have been performed to investigate posttranslational modifications of EPO with a focus on the glycosylation and sialylation patterns.[8,22] All hEPOs are cleared from circulation through a hepatic galactose receptor demonstrating the importance of sialylation. Pharmaceutical companies have addressed this issue by selective purification toward a high degree of sialylation or genetic manipulation of the peptide backbone to increase the glycosylation. Glycosylation of the protein core of EPO prolongs the hormone's half-life in the circulation and is therefore essential for biologic activity.[23,24] Darbepoetin alpha (NESP) has been shown to have an increased half-life as well as an increased biologic activity in vivo resulting from the excess of sialic acids when compared with endogenous hEPO. The glycosylation pattern strongly depends on the cells used for the protein expression. The biosynthesis of glycans is species-, tissue-, and cell type-dependent, but the culture conditions may also contribute to the microheterogeneity, resulting in a large diversity of glycan structures.[8,25–28] Consequently, endogenous EPO as produced in human kidney cells is modified differently compared with rhEPOs, which are commonly produced in Chinese hamster ovary or baby hamster kidney cells.[29] In contrast, epoetin delta is engineered in cultures of the human fibrosarcoma cell line HT-1080 by homologous recombination

TABLE 1. Overview of Structural and Functional Erythropoietin Variants[15–21,76,118]

Compound Name	Group	Molecular Weight (kDa)	Remarks
Epoetin alpha/ beta	rhEPO	30–34	Produced in hamster ovary cell lines, identical amino acids sequence with hEPO, vary only in their glycosylation pattern
Epoetin delta	rhEPO	26–32	Identical amino acids sequence with hEPO, lacks N-glycolyl-neuraminic acid (Neu5Gc), produced in human fibrosarcoma cell line, pharmacokinetic profile similar to Epoetin alpha/ beta
Epoetin omega	rhEPO	34	Produced in hamster kidney cells
Darbepoetin	rhEPO	37	Primary structure differs in five positions compared to hEPO, hyperglycosylated rhEPO (asparagine residues at positions 30 and 88 are additionally glycosylated), also known as novel erythropoiesis stimulating protein (NESP)
Continuous erythropoiesis receptor activator (CERA)	rhEPO	60	Polethylenglycol-linked epoetin beta, prolonged half-life and increased in vivo biological activity compared with epoetins
Carbamoylated rhEPO (CEPO)	rhEPO	40	Suggested to bind to EPOR-β common receptor heteromer, reduced risk for thrombosis as compared to rhEPO
Carbamoylated darbepoetin (Caranesp)	rhEPO	40	
Asialo-EPO	rhEPO	30–34	Desialated rhEPO
EPO-S100E	rhEPO		
HBP (Helix B peptide), pHBSP (pyroglutamate Helix B surface peptide)	EPO mimetic peptide (EMP)	20 amino acids (aa) 11aa	Suggested to bind to EPOR-β common receptor heteromer
Epopeptide AB	EMP	17 aa	
Hematide (synthetic PEGylated peptidic erythropoiesis stimulating agent)	EMP	20 aa, 5 kDa	Pegylated peptide unrelated to EPO, phase III clinical trials, significant elevations of hemoglobin and hematocrit values
K-11706/K7174	GATA-2 inhibitor		Induces EPO gene expression
Compound A, TM 6008/TM 6089, FG-2216/FG-4592, Betahydroxybutyrate	Hypoxia-inducible factor (HIF)-stabilizer		Small molecules inhibitors of propyl hydroxylases, induce EPO gene expression, some of them orally active peptides

and gene activation, unlike old forms of rhEPOs that are produced in other mammalian cells. Therefore, epoetin delta is considered to have a human-type glycosylation profile.[30]

Endogenous Production, Biochemical Action, and Applications of Recombinant Human Erythropoietins

hEPO stimulates proliferation and differentiation of erythroid precursor cells and red blood cell production in the bone marrow. Thus, it is mainly involved in the regulation of the oxygen supply of the human body. Endogenous hEPO production is regulated predominately by the oxygen demand of the organism. Hypoxia leads to a rapid increase in the transcription of the EPO gene in the EPO-producing cells, resulting in increased hEPO serum levels within 2 hours. It is presumed that a transmembrane hemoprotein acts as an oxygen sensor and that the deoxygenated form of this protein activates a transcription factor, which in turn enhances EPO gene transcription in the nucleus. Apart from hypoxia, agents such as human growth hormone, adrenocorticotropic hormone, interleukin-3, angiotensin II, and adrenaline are also capable of increasing endogenous hEPO production.[31,32]

The understanding of the molecular mechanisms in control of the EPO gene expression in vivo remains deficient. The expression of the EPO gene is under the control of several transcription factors. GATA binding protein 2 (GATA-2) and nuclear factor kappa-light-chain-enhancer of activated B cells act on the 5′ promoter and inhibit EPO gene expression.[33] GATA inhibitors partially reverse the inhibition of EPO gene expression by proinflammatory cytokines.

The hepatic hypoxia-inducible EPO enhancer is a 50-bp element located at the 3′ terminus of the EPO gene and contains at least two transcription factor-binding sites. The proximal site of the EPO enhancer binds to the hypoxia-inducible factors (HIFs),[34,35] which are composed of an O_2-labile α-subunit and a constitutive β-subunit.[36] HIF-2 (rather than HIF-1) appears to be the main physiological transcription factor inducing EPO gene expression in hypoxia. HIF-2α predominates in the EPO-producing peritubular fibroblasts in the rat kidney, whereas HIF-1α is mainly present in renal tubular cells and has different cell-protective functions.[37]

EPO gene expression is suppressed in normoxia because HIF-α undergoes prolyl and asparaginyl hydroxylation. Prolyl hydroxylation causes binding of the Von Hippel- Lindau protein E3 ubiquitin ligase complex to HIF-α, which is then degraded in the proteasome. Asparaginyl hydroxylation of HIF-α reduces its transcriptional activity by preventing binding of the coactivator p300. There have been three HIF-α prolyl hydroxylases and one asparaginyl hydroxylase identified in human tissues. These enzymes require O_2, iron (Fe^{2+}), and 2-oxoglutarate to express their catalytic activity. Accordingly, HIF-α hydroxylation can be prevented by iron depletion or by the application of 2-oxoglutarate-competing analogs.[38]

A major concern is the tumorigeneic potential of the HIF stabilizers because HIFs activate several genes coding proteins (eg, vascular endothelial growth factor) that promote tumor growth. It will be of major importance to develop HIF stabilizers that allow for a tissue-specific delivery of the drugs. For example, EPO is produced in the eye, and increasing this production could worsen diabetic retinopathy because EPO promotes angiogenesis.[39] Abuse of HIF stabilizers that promote EPO expression cannot be excluded in athletes and warrants further attention.

The main action of both endogenous EPO and rhEPOs is to stimulate the proliferation and differentiation of progenitor cells in the bone marrow toward functional erythroblasts.[40,41] The subsequent maturation requires 5 to 9 days under normal physiological conditions. The duration of the erythropoietin-induced effects at the gene level is greater than that of any hematologic changes associated with the misuse of rhEPOs.[42]

Many patients with chronic kidney disease have benefited from the use of rhEPOs to correct severe anemia. However, recent mortality data suggest that treating patients with chronic kidney disease to achieve a hemoglobin level greater than 13 g/dL may be harmful. Although studies demonstrate superior quality of life and functional status, the impact of venous thrombotic events continues to be debated.[43]

rhEPOs have also been trialed in preclinical and clinical studies for their therapeutic potential in human brain diseases such as acute ischemic stroke, chronic schizophrenia, and chronic progressive multiple sclerosis. EPO and its receptors are expressed in the nervous system and peripherally administered EPO crosses the blood–brain barrier, stimulates neurogenesis, neuronal differentiation, and activates brain neurotropic, antiapoptotic, antioxidant, and anti-inflammatory signaling. Nevertheless, particularly considering the high dose of EPO required to pass the blood–brain barrier, the use of EPO as a neuroprotective/neuroregenerative treatment strategy in human brain disease requires close clinical and laboratory monitoring of patients.[21]

Anemia in patients with solid tumor cancer is approximately 40%, whereas in hematologic malignancies, it is almost twice that.[44] The frequency of anemia increases with the duration of chemotherapy. Use of rhEPOs and erythropoiesis-stimulating agents improves erythropoiesis and reduces transfusion needs. However, especially when the hematocrit (Hct) level is normal, adverse effects such as greater mortality from serious cardiovascular and thromboembolic events and tumor progression have been observed, which led the Food and Drug Administration in 2007 to issue a relevant warning. Nevertheless, neither erythropoietin nor its receptor are oncogenic and do not stimulate tumor proliferation.[45]

Anemia is also common in patients with chronic heart failure and erythropoiesis-stimulating proteins are used for its treatment, without major concerns such as a higher mortality rate or more adverse events, whereas the beneficial effect of reduced hospitalization in patients with heart failure is reported.[46]

Because EPO increases the amount of oxygen that is carried to the muscles, rhEPOs have also been embraced as an ergogenic aid in endurance sports,[5,47,48] whereas anecdotal reports point to the use of rhEPOs in power or anaerobic sports/disciplines. However, apart from increasing the Hct, the excessive use of EPO or rhEPOs can also result in serious side effects such as red cell aplasia and heart failure. Their use increases the risk of cardiovascular problems, including

cardiac arrest, seizures, arrhythmia or strokes, hypertension and hypertensive encephalopathy, congestive heart failure, vascular thrombosis or ischemia, myocardial infarction, and edema. Adverse reactions can also include hypotension, fever, chest pains, nausea, and myalgia.[49,50] EPO also appears to be implicated in angiogenesis.[51] EPO withdrawal may result in neocytolysis.[52]

rhEPOs that have a high molecular weight and a complex three-dimensional structure are produced by living organisms and are therefore inherently heterogenous and, importantly, have the potential to be immunogenic. Impurities derived from the host cell line, the complex growth media required, and the purification processes are inevitable. For example, 11 epoetin alpha products from three manufacturers were analyzed and variable isoform patterns were found even between samples from the same manufacturer and the in vivo bioactivities ranged from 71% to 226% of the standard used. Of major concern is the fact that a few preparations contained unacceptable levels of bacterial endotoxins.[43] For all these reasons, authorities must develop a regulatory framework such as a pharmacovigilance plan that will collect safety data and clearly identify exposure to specific biosimilars.

rhEPO misuse was suspected in almost 20 deaths in 4 years in European cyclists.[53] As early as 1972, the International Olympic Committee (IOC) recognized the dangers and threat of blood doping in general and in 1987, the class of peptide hormones was added to the IOC's List of Prohibited Classes of Substances and Methods (List). However, because misuse of rhEPO in sports was undetectable, EPO was not included in the IOC's List until 1990.

Analytical Milestones in the Fight for Recombinant Human Erythropoietins Detection in Doping Control

The challenge of detecting EPO misuse has prompted several proposed strategies.[54] The physiological and biochemical effects of its administration may be used to suspect the application of this hormone and is the basis of the so-called indirect methods. These use the measurement of certain hematologic and serum parameters and their comparison with population cutoff values. Hemoglobin, for example, is attractive because it reflects the oxygen-carrying capacity of the blood, which is ultimately what all forms of blood doping seek to modify.[55] The hemoglobin assay is internationally standardized and has better analytical characteristics than those of the Hct.[56] In 1995, the International Ski Federation introduced hemoglobin limits for competing athletes. Skiers were excluded from competition if their hemoglobin exceeded 175 g/L for men and 155 g/L for women. In 1999, the International Cycling Union and International Biathlon Union introduced Hct limits for competing athletes. Cyclists were unable to compete for 14 days if Hct values were greater than 50% for men and 47% for women and reticulocytes (ret) above 2%, respectively. Recently, the International Cycling Union has introduced free plasma hemoglobin to combat the use of synthetic hemoglobin (hemoglobin-based oxygen carriers). These values do not represent definitive proof of rhEPO use that could lead to the athlete's sanctioning but are biochemical findings that raise the suspicion of doping and are used to protect the athlete's health by excluding him or her from competition ("health" tests).[57–60] Moreover, apparently some athletes consume several products or drugs concomitantly such as volume expanders, growth hormones, interleukin 3, and insulin. Because the physiological and/or hematologic consequences of these associations remain unknown, additional difficulties occur in the indirect detection of rhEPO.

The first report on direct rhEPO detection, based on the observation that the electric charge of the rhEPO molecule is less negative than that of endogenous EPO, dates back to 1995.[61] In 2000, a brief communication in *Nature* was published describing the development of an analytical procedure comprising isoelectric focusing followed by chemiluminiscent immunodetection of blotted EPO for detecting rhEPO in urine.[62]

The IOC launched the EPO2000 research project in 1999. The scientific collaborators who participated in this work came from Australia, Canada, China, France, and Norway. Funds of US $2 million were provided. As a result, during the Sydney 2000 Olympic Games (OG), the first pilot scale tests for rhEPO were conducted using combined blood and urine tests based on the detection of markers of altered erythropoiesis in blood[63] and the detection of isoelectric profiles of EPO in urine, which can differentiate natural and administered recombinant hormones (the Lasne et al method).[7] The blood test used in the Sydney OG was based on two statistical models, the current ON model and the recently discontinued OFF model that are useful indicators of rhEPO use by athletes. The component variables of the original ON model were Hct, % ret, serum EPO, percent macrocytes (%Macro), and soluble transferrin receptor (sTfr), whereas the OFF model uses only the first three variables. Follow-up studies that included more subjects, different ethnic groups, and various doses of rhEPOs confirmed the original findings and enabled the development of refined "second-" and "third generation" ON and OFF models.[64,65]

Many parameters involved in the previous equations were already known to be affected by changes in erythropoiesis. The new % ret parameter was hypothesized to contribute to the detection of subjects with an artificially induced change in the rate of erythrocyte production, because both the number of ret and their mean cell volume were known to be affected by erythropoiesis.[66] The Lasne et al urine test involves isoelectric focusing (IEF) of the retentate from ultrafiltered urine. The double-blotting process was developed to solve the problem of nonspecific detection of various urinary proteins in the focused retentate by classic immunoblotting. Sufficient sensitivity was achieved using amplified chemiluminiscent detection after the blotting membrane was treated with dithiotreitol.[7] To recognize an adverse analytical finding during the Sydney OG, the two methods had to correlate.

In the 2002 Salt Lake City Winter OG, a different detection scheme was adopted. A blood screening test measuring hemoglobin (cutoff limits 175 g/L for men and 160 g/L for women) and % ret (cutoff limit 2%) was used followed under certain circumstances by a urine test according to the Lasne et al method. Athletes with abnormal hematologic values were required to return for a repeat blood screen on the day of their next competition. In addition, a maximum of 20%

of the field of athletes were screened on the day of each competition. Athletes with a suspicious blood screen were required to provide a urine specimen. The decision for a possible "no start" was to be made by each International Federation. Approximately 100 athletes at the Salt Lake OG who had abnormal values from the blood screening passed the Lasne et al urine EPO test. A new form of rhEPO, darbepoietin alpha, was used by athletes and detected with the Lasne et al method before it was listed as prohibited. Three cross country skiing medalists who tested positive for darbepoietin were subsequently stripped of their medals. The athletes appealed the decision to the Court of Arbitration for Sport, which accepted that the test was scientifically valid.

In 2003, a report for WADA by the scientists Thormann and Peltre concluded that the Lasne et al urine EPO test was the only existing test capable of detecting and quantifying urinary rhEPOs. Since then, this test has become the official WADA method for the detection of rhEPOs misuse and has been applied as part of the official doping control program at the Athens and Beijing OG as well as in all major athletic events. Moreover, since the Athens OG, testing has been extended to anaerobic sports and disciplines. The screening of various parameters in blood as indirect evidence for doping has not been completely abandoned and continues to offer alternatives in the fight against doping. Currently, several International Federations continue to perform "health tests."

Although the Lasne et al method is now used by the majority of the WADA-accredited laboratories, unofficial data indicate that approximately 10% to 15% of all EPO tests show undetectable profiles.[67] Moreover, the method recently met with criticism, and controversy has arisen among various research teams.

The Direct Method for Recombinant Human Erythropoietin Misuse

As previously mentioned, rhEPOs have different isoelectric profiles from the endogenous hormone (lower negative median charge). Endogenous hEPO presents 10 to 15 isoforms (pI 3.8–4.7), whereas epoetin alpha presents five isoforms (pI 4.4–5.1), epoetin beta 5 isoforms (pI 4.4–5.1) plus one more basic and darbepoietin alpha 5 isoforms (pI 3.0–3.9). The method needs a considerable amount of urine in comparison to usual analytical methodologies applied for doping control (2–5 mL of urine). Twenty milliliters of urine are ultrafiltrated to concentrate the urine 200 to 1000 times using Tris-HCl pH 7.4 to inactivate aspartic proteases. A WGA sepharose treatment was originally proposed by Lasne et al to reduce the protein content of the final retentate and avoid arc-shaped bands after electrophoresis. To prevent EPO degradation by proteases, a general protease inhibition cocktail is used, which is considered more efficient compared with heating or treatment by pepstatin. The retentate is subjected to IEF (the filtrate can be used for routine screening of anabolic steroids, stimulants, diuretics, etc). The EPO loading of the gel should be 1500 IU/mL and for rhEPO 600 IU/mL (Tris/HCl, pH 7.4 is used for dilutions). A polyacrylamide gel containing 7 M of urea, ampholytes (6–8 ampholytes as a catholyte and H_3PO_4 as an anolyte) and sucrose is used. After the IEF run, immunoblotting follows using an AE7A5 anti-EPO primary antibody, which binds to an epitope within the first 26 aa of the molecule. Treatment with dithiothreitol (reducing agent) directly after the semidry transfer increases sensitivity up to threefold. Double blotting to prevent nonspecific binding to various urinary proteins using a biotinylated antimouse IgG secondary antibody with chemiluminescent substrate is required. More specifically, after probing with the primary antibody, the membrane with the blotted proteins is assembled with a second blank membrane and undergoes a second blotting under acidic conditions. In this way, the primary antibody is desorbed and transferred to the second membrane, whereas the antigen and the interfering proteins remain on the first membrane. The second membrane is then probed by the secondary antibodies without the risk of nonspecific binding.[68] The sensitivity achieved with the Lasne et al method is of approximately 0.2 mIU per band and the minimum concentration of EPO to be detected in urine is 0.4 IU/L.[7]

Criteria for Declaring an Adverse Analytical Finding

From an analytical point of view, the detection of rhEPO in human body fluids is challenging for various reasons. rhEPO levels in human serum/plasma and urine are very low, usually in the low fmol range. The detection of rhEPOs cannot be based solely on the presence of a unique band. Endogenously produced EPO also extends its isoform profile through some of the rhEPOs pI values. An approach to develop objective criteria to analyze the electrophoretic image obtained was developed by Lasne et al,[69] whereby the image is converted into numeric data corresponding to the positions and relative intensities of the bands. Classification of EPO profiles was performed accurately, using discriminant analysis, thus preventing false-positive interpretations and improving the detection of rhEPO. WADA, in collaboration with a group of experts, produced a technical document (TD)[70] to harmonize the declaration of an adverse analytical finding by accredited doping control laboratories. Subsequently, this TD has had to be modified to include the development of criteria to accommodate both new evidence and the introduction of novel recombinant products. In this TD, the establishment of certain objective criteria for the identification of the presence of the recombinant molecules was presented. The TD comprised two sets of criteria: 1) those that describe the quality of the IEF image in relation to the background and the clear assignment of band numbers; and 2) those to identify epoetin alpha, beta, and darbepoietin alpha. The latter are based on the assignment of acceptable, consecutive bands in the basic or acidic area, the intrarelative intensity of these bands, and the relative intensity of these bands with regard to the bands in the endogenous area. This means that to a certain extent, the actual positivity criteria take into consideration the endogenous EPO production rate, which differs from one subject to another. Furthermore, a serious concern would be whether subjects with a naturally elevated endogenous EPO production but misusing rhEPOs could be considered negative.[67] It should be recalled that endogenous EPO production will be depleted when administering rhEPOs through a negative feedback mechanism.

The positivity criteria applied for darbepoietin alpha were challenged when an excretion study, after a single subcutaneous injection of 40 mg ARANESP® [illegible] to a healthy individual, was published.[67] Compared with epoetin beta, darbepoietin alpha is less suitable to doping than epoetins because of its longer detection window (approximately 7 days). In general, the timeframe for the direct detection of rhEPO isoforms is relatively short with the Lasne et al method, approximately 24 to 72 hours after the last rhEPO administration, depending on the dosage and the route of administration. As demonstrated in the excretion study, the bands of darbepoietin were more intensive than any endogenous EPO bands. Consequently, endogenous bands may disappear as a result of the possible feedback regulation of endogenous EPO production. Nevertheless, in a few intermediate samples of the excretion study, the most intense band corresponded to a band in the endogenous portion of the gel. Physiologically, this may eventually be explained by a sudden increase of endogenous EPO production (eg, hypoxia after high-altitude exposure or strenuous physical activity). The authors claim that in the case of darbepoietin alpha, the last positivity criteria of the WADA TD[70] is subject of debate. The most relevant information should not be the band intensity ratio, but the position and the specific distribution of the bands in the most acidic area of the gel, especially because atypical or even doubtful profiles in the very acidic and specific domains of darbepoietin alpha in a gel have never been reported. WADA have acknowledged this by issuing revised guidelines in 2009.[71]

This latest version of the WADA TD also refers to epoetin delta reducing earlier concerns raised that epoetin delta might be undetectable in the current IEF urine test.[72] The matrix-assisted laser desorption/ionization–time of flight mass spectrum of the molecule showed that the molecular weight of epoetin delta is within the range of the other epoetins and therefore probably not as similar to hEPO as expected from the production in human cells.[30] Nevertheless, on September 28, 2007, the French sports newspaper *L'Equipe* reported that the French antidoping laboratory had detected Dynepo® (epoetin delta) in the urine of Michael Rasmussen during the 2007 Tour de France. As shown by various researchers,[30,73] the IEF profile of epoetin delta, in which more acidic isoforms can be observed with respect to Chinese hamster ovary-derived marketed rhEPOs,[7,74,75] differs from that of endogenous EPO (from either urine or plasma). With the rigorous structural analysis of epoetin delta completed, it can be concluded that when compared with other recombinant EPOs, the more acidic bands present in IEF are the result of a higher degree of sialylation.

Included in WADA's TD 2009 version is the identification of Continuous Erythropoiesis Receptor Activator (CERA), a new third-generation erythropoiesis-stimulating agent recently linked with misuse in endurance sports resulting in several adverse analytical findings at 2008 Beijing OG. Specific criteria regarding the presence of bands in the strong basic area using the Lasne et al method for pronouncing an adverse analytical finding are described.[71] An enzyme-linked immunosorbent assay has been designed and validated for detection of CERA in serum samples. This test has been proposed as a high-throughput screening method for antidoping purposes combined with the classic IEF test as a confirmatory assay.[76]

Criticism of the Direct Method for Recombinant Human Erythropoietin Abuse

The validity of doping sanctions based on the Lasne et al method was first challenged successfully in court in 2005 by the Belgian triathlete Rutger Beke. According to published press information, 10 months after his suspension from competition, the Flemish Disciplinary Commission accepted that his sample had become degraded by bacterial contamination and, as a result, the substance identified by the laboratory analysis as pharmaceutical rhEPO was an unrelated protein. Similarly, a few cases have been reported in which the profile obtained in the first analysis of the sample (the so-called screening) was different from the one obtained later in a second confirmatory analysis. The causes remain to be confirmed, although bacterial contamination of the urine has been claimed as one of the likely causes.[66]

A possible explanation for Beke's case is the potential effect of strenuous exercise on urinary EPO, leading to the so-called atypical IEF profiles (endogenous EPO shifted toward the basic area where rhEPO migrates). This provoked a controversy among scientists.[77–81] A brief report by Beullens et al described a case in which urine samples, obtained from an endurance athlete, who had not used any rhEPOs, were analyzed by the Lasne et al urine test immediately after a strenuous interval training session. In some samples, these authors claimed bands had migrated like epoetin beta isoforms during IEF and was attributed to strenuous exercise.[79] The authors also pointed out that the false-positive detection of epoetin beta may be linked to the extent and type of proteinuria. They argued that the extent of proteinuria correlates more with the intensity than the duration of exercise and has a half-time decay of approximately 1 hour. To avoid such false-positives, they suggested sampling before or at least 1 hour after exercise, which is particularly important for the few athletes who present with pronounced exercise-induced proteinuria.[79] It should be mentioned that Beullens et al did not identify the interfering protein they reported. In contrast, Reichel reported a protein, identified as zinc-alpha-2-glycoprotein, that appears in urine after strenuous exercise, which clearly does not migrate close to the EPO glycoforms.[82] However, the quality of the IEF image presented by Beullens et al was relatively poor. It was counterargued that such a result would not only have been categorically rejected from any interpretation in an antidoping control,[78] but that strict application of the identification criteria would have also led to a negative report.[77] Nevertheless, having conducted the test by applying the same criteria, the authors reached a conflicting conclusion, declaring the result as positive,[80] showing that despite the guidelines of the WADA TDs, the interpretation of the Lasne et al method results remains rather subjective. In a recent study from the Suisse Doping Control Laboratory, it was shown that atypical urinary EPO patterns caused by supramaximal brief exercise did not fulfill WADA's three conservative criteria mandatory for reporting an adverse analytical finding.[83]

In 2006, some of the alleged technical weaknesses and risks of the Lasne et al method were summarized.[84] The concentration of urea used for denaturation and isoelectric focusing (7 M) is regarded as inadequate for full protein

denaturation. The commonly used protocol for IEF applies a concentration of 9.5 M urea.[85] It could be argued that under such conditions, additional interferences may be observed. Furthermore, the ionic strength used in the Lasne et al method is too low to guarantee the dissociation of possible ionically bound complexes formed with highly charged molecules such as EPO. The fact that no thiol agent or any other reduction chemical is applied during the entire protein preparation may result in oxidative complex formations. However, the most important criticism relates to the primary antibody used in the Lasne et al method. The monoclonal antibody AE7A5 is not specific for rhEPO, or for EPO in general, and can crossreact with a high number of other proteins such as eukaryotic or bacterial. Therefore, obligatory tests for proteolytic, glycolytic, or modifying activities in the urine are suggested. Finally, bleeding from the urothelial surface can interfere, because endogenous human serum EPO focuses in a similar band pattern and the same pH range as urinary rhEPOs.

"Unstable" urines have also caused recent concern. Previous studies have demonstrated that proteases such as arysulfatase, sialidase, subtilisin, trypsin/chemotrypsin, and papain in urine samples are responsible for elimination of endogenous and rhEPOs signals.[75] The addition of respective competitive substrates such as p-nitrocatechol sulfate and sialyllactose[86] or a combination of antibiotics, antimycotic substances, and protease inhibitors has been proposed as a solution.[87]

In view of these concerns and seeking to clarify the situation, in May 2007, WADA updated its technical document and a stability test was introduced in which the sample is incubated in the presence of rhEPO and NESP to check for any change in the profiles.[88]

Early in 2010, the first case of tampering urine samples with proteases was substantiated in the Court of Arbitration for Sport and involved Portuguese cyclists. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis combined with liquid chromatography coupled to tandem mass spectrometric analysis, the presence of proteases Bacillolysin was detected. These proteases destroy glycoproteins introduced into the urine samples, rendering analysis for rhEPO impossible.[89]

In 2008, Lundby et al,[90] who investigated the detection power of the Lasne et al test as well as the variability between two WADA-accredited laboratories, reported poor correlation in test results between the two laboratories. Moreover, after the initial rhEPO-boosting period, the power to detect rhEPO misuse during the maintenance and post periods appeared minimal. Enlargement of this timeframe for rhEPO detection is only possible by implementation of additional blood and/or biochemical parameters. Nevertheless, Lundby's results have been vigorously questioned[91–93] by the two WADA-accredited laboratories involved, claiming that different methods were applied, although, according to Lundby, this was not what had been agreed in the study protocol and their claims came to light after the publication of Lundby's paper.[94]

Alternative Approaches to the Direct Recombinant Human Erythropoietin Detection Method

A new electrophoretic approach using sodium dodecyl sulfate–polyacrylamide gel electrophoresis mobilities of target analytes has recently been presented.[95] The urine samples were concentrated for analysis as described elsewhere.[7] Using two internal standards (darbepoietin alpha and recombinant rat EPO), the assay provides a tool, which allows for the calculation of relative mobility values for endogenous urinary EPO and recombinant epoetins (eg, epoetin delta) and, thus, the distinction of these analytes in doping control samples. An epoetin delta excretion study was conducted in one individual and urine samples were analyzed and compared with those from a reference group of 53 healthy volunteers. Significant discrimination of endogenous urinary and rhEPO molecules was observed. A clear differentiation was accomplished over a period of 4 days postadministration of a single injection of 50 IU/kg body weight. Hence, the method has been accepted by WADA[71] as a useful screening procedure or as a complementary confirmation tool to the established IEF assay in doping control, especially in light of the constantly increasing number of EPO biosimilars entering the pharmaceutical market.

However, the sensitivity of sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the detection of CERA in urine is markedly decreased the result of the limited excretion of this rather large molecule (approximately 60 kDa) (Table 1). SARCOSYL is a methyl glycine-based anionic surfactant with the property to bind only to the protein part of CERA, but not to its polyethylene glycol chain, whereas SDS binds to both. Exchanging SDS with SARCOSYL in the sample and running buffers is reported to solve the problem without compromising for the other rhEPOs.[96]

As mentioned, information derived from blood analyses can assist in the detection and/or deterrence of blood doping. Several hematologic indices (number of red blood cells, blood hemoglobin concentration, Hct, ret count, number of hypochromic macrocytes) and biochemical parameters (serum EPO and sTfr concentrations) change significantly after rhEPO intake.[97,98] Usually cutoff values are established at the 95th or 99th percentile of the normal population based on the mean population values. Depending on which set of population-derived statistics are used, these thresholds overlap the population distribution values for healthy males so that approximately 1% to 5% of the male population would exceed these values. Possible reasons include the use of blood doping by subjects within the cohorts (ie, true-positives), hematologic abnormalities, and sampling error. Because altitude is known to affect our blood analysis, it would be advisable that sport federations included blood values obtained from athletes while located at a high altitude.[65] Extensive investigation led to a proposal of mathematical models using two or three parameters to detect an altered erythropoiesis. The use of more than one parameter increases specificity, reducing the possibilities of a false-positive. Those multiparametric models are based on common hematologic techniques and clinical analyzers. Nevertheless, such a method would still be considered by many antidoping and legal authorities as inadequate to confidently confront an athlete with an accusation of doping.[42]

For an athlete who provides a blood sample showing suspicious changes from historical values, a subsequent step may be to conduct an in-depth hematologic evaluation to identify any congenital or acquired hematologic disorders. Such an evaluation should comprise a red blood cell count,

iron status evaluation, bilirubin, lactate dehydrogenase, serum iron, total iron-binding capacity, serum ferritin, sTfr, and serum EPO levels.[99] Comparing an athlete's hematologic values against his or her own historical baseline rather than population-derived thresholds enhances the ability to detect blood doping. The so-called hematologic passport offers an efficacious tool to identify hematologic fluctuations that are suspected of having been caused by prior blood doping but which fall below the absolute level associated with a "no start." This information could be used by antidoping agencies when compiling lists of athletes to be target-tested in the lead-up to future competitions.[65] Recently WADA issued a technical document describing blood analytical requirements for the athlete biologic passport.[100]

When comparing results or reviewing longitudinal studies, several complicating factors must be considered, including the inherent variability in immunologic methods, the potential changes in batch-to-batch antibody reagents, and the lack of harmonization for the standardization of the assays[101] such as for sTfr or % ret.[102] Of particular interest for the indirect models discussed is the reliability of immunologic assays for serum EPO and sTfr. Several comparisons of immunoassays for EPO have been published.[64,103–105] However, as a result of the variability of enzyme-linked immunosorbent assay results for serum hEPO and sTfr, those parameters are suitable for screening purposes but not for confirmation testing.[106] The outcome of studies in this field is that an exhaustive validation of the methods used for comparison purposes is of paramount importance, including intralaboratory, interlaboratory, and intertechnique variability.[64,107–109]

Some investigators have attempted to enrich the screened parameters for altered erythropoiesis with the determination of messenger RNA of erythroid target genes such as β-globin, ferritin-light chain, and ornithine decarboxylase antizyme by quantitative polymerase chain reaction.[42,110] β-Globin is considered a selective marker of erythroid activity and its expression (upregulated during erythropoiesis stimulation) appears to change most significantly after rhEpo intake as described by Magnani et al.[110] Moreover, considering its occurrence in the reticulocyte library, the estimated β-globin level was 30% of total mRNA and represents the major reticulocyte transcript. As for ferritin-light chain, the red cell pool incorporates iron during erythroid expansion. The use of rhEpo may cause a change in iron metabolism. The mRNA content of ferritin-light chain, a protein implicated in iron metabolism and storage, may prove to be a useful marker of exogenously altered erythropoiesis. These markers have previously been tested on a human erythroleukemic TF1 cell line treated with rhEPO, which provides an in vitro model of erythroid differentiation.[42] The use of transcriptome data will further help researchers to define additional genes of interest.[111] The recent development of a real-time polymerase chain reaction, a rapid, reliable, and valid method, could constitute a new perspective in blood antidoping research and such an approach may be an additional weapon in the war against doping.

Another study surfaced recently, suggesting that changes in the asymmetric dimethylarginine, dimethylarginine dimethylaminohydrolase, and nitric oxidesynthase system reflecting endothelial damage could be used as indirect markers for rhEPO administration in doping control. The predictive value of four proposed new markers in urine (asymmetric dimethylarginine, symmetric dimethylarginine, arginine, and citrulline) for rhEPO misuse was tested. These preliminary results indicate that an indirect approach may be used as a form of prescreening of urine samples to decrease the number of samples with a low probability of rhEPO misuse and saving costs and human workload.[112]

In doping control, analytical methods leading to a direct proof of substance abuse are preferred. To develop a faster and more reliable method for the direct detection of rhEPOs and future derivatives, researchers have focused on various mass spectrometric (MS) strategies. Matrix-assisted laser desorption/ionization mass spectrometry applying a high-resolution time-of-flight mass analyzer in the linear mode was used in some studies for the analysis of intact EPO molecules[113,114] as well as epoetin alpha, epoetin beta and delta, and darbepoietin alpha.[115] As a result of the high amount of heterogeneous sugar moieties, only approximate average masses were determined. Detection limits for the highly purified, intact glycoproteins were achievable in the low fmol range (25–50 fmol) using a sample preparation method by applying a hydrophobic sample support (DropStop1) as a matrix-assisted laser desorption/ionization target surface.[115] Lately, a new method has been described for the identification of darbepoietin in human plasma using immunoaffinity separation and enzymatic digestion before tandem mass spectrometric analysis.[116] The fact that in equine plasma, a similar method has led to differentiation and identification of rhEPOs[117] indicates that these early results are very promising for the development of highly sensitive detection methods for a direct identification of rhEPO after enrichment from human body fluids.

Hematide (Table 1), with a half-life of more than 7 days after subcutaneous application, may also be misused by athletes as a result of its erythropoietic properties. This EPO analog does not crossreact with EPO antibodies, and thus conventional doping control assays for EPO would not detect the misuse of Hematide. Consequently, MS-based assays, probably tandem mass spectrometric analysis mass spectroscopy techniques, could enable the detection of Hematide or degradation products in urine and/or blood samples. However, currently no assay is available that definitely detects Hematide or its degradation products in doping control samples.[118]

Finally, the structure of HIF stabilizers such as FG-2216 has not been disclosed and no pharmacologic studies on their metabolic fate have been published. Therefore, doping control assays cannot as yet be developed. Unique mass spectrometric fragmentation behavior, which was observed in a series of related compounds, should allow sensitive screening for such drugs and their metabolites in blood and urine doping control specimens using, eg, the neutral loss scan feature of triple quadrupole mass analyzers. Suspicious signals derived from experiments, which yield product ions that eliminate 11 Da, would then require follow-up studies to determine whether they originate from prohibited compounds related to HIF stabilizers.[118]

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