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Basic analytical methods for identification of erythropoiesis-stimulating agents in doping control

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The design of new erythropoiesis-stimulating agents for clinical use necessitates constant development of methods for detecting the abuse of these substances, which are prohibited under the World Anti-Doping Code and are included in the World Anti-Doping Agency (WADA) prohibited list. This review integrates and describes systematically the published data on the key methods currently used by WADA-accredited anti-doping laboratories around the world to detect the abuse of erythropoiesis-stimulating agents, including direct methods (various polyacrylamide gel electrophoresis techniques, enzyme-linked immunosorbent assay, membrane enzyme immunoassay and mass spectrometry) and indirect methods (athlete biological passport). Particular attention is given to promising approaches and investigations that can be used to control prohibited erythropoietins in the near future.
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I. Introduction

The modern stage of elite sport development is characterized by ever-increasing sporting achievements, sometimes transgressing the limits of human nature and putting an athlete health at risk. Elite athletes are exposed to physical and psychological stress that virtually exceeds their adaptive capacities, and the use of new training schemes and programmes does not solve this problem. Therefore, approaches to the improvement of physical traits and enhancement of athletic performance are looking for doping agents. In recent years, so-called haematopoietic stimulants have gained wide popularity among athletes. Erythropoietin (EPO) is one of such stimulants. Erythropoietins promote the formation of red blood cells (erythrocytes), thus increasing the oxygen-carrying capacity of the blood and significantly stimulating metabolism in the body.

Erythropoietin is a glycoprotein hormone secreted mainly by the kidney and, in insignificant amounts, by the liver. In 1985, Lin *et al.*[1] cloned the human EPO gene. Later on, the gene was expressed in Chinese hamster ovary cell lines (CHO cells). This provided an opportunity to produce EPO for clinical use on the industrial scale. In 1989, the first

recombinant human erythropoietin (rEPO) — Eprex — was approved for the treatment of patients with various forms of anemia. Erythropoietin causes an increase in the haemoglobin concentration in the blood, thus enhancing oxygen transport and endurance performance, resulting in the application of EPO as a doping means.

In 1990, the International Olympic Committee (IOC) included EPO in the list of prohibited substances.[2] The first direct method for the detection of EPO in urine was reported in 2000.[3] This method was capable of distinguishing between endogenous and exogenous erythropoietins based on isoform profiles determined by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) followed by double immunoblotting. The design of new erythropoiesis-stimulating agents (ESAs) and their approval for marketing as pharmaceutical products triggered the development of methods for the detection of prohibited haematopoietic stimulants. In 2001, the rEPO preparation darbepoetin was marketed under the trade name NESP; epoetin delta (Dynepo™) was approved for use in 2002 and was marketed in 2007; Mircera (CERA, methoxy polyethylene glycol–epoetin beta), a continuous erythropoietin receptor activator, was launched in 2007. The new PEGylated EPO-mimetic peptide, known as peginesatide or Omontys™, was finally prohibited for production because of increased lethality in volunteers in clinical trials.[4,5] In recent years, particular attention has been given to EPO-Fc hybrid glycoproteins consisting of human EPO and the Fc fragment of the immunoglobulin heavy chain[6,7] and exhibiting erythropoiesis-stimulating activity comparable with that of the other known recombinant EPOs. However, data on their clinical use are lacking.

At the present time, not only urine but also blood samples are used as a biological material in doping control analysis since Mircera, peginesatide and EPO-Fc are more easily detected in blood serum or plasma.[8–11] In order to decrease the assay time, the enzyme-linked immunosorbent assay (ELISA) technique was developed for rapid screening of Mircera in the blood serum.[8] The efficient detection of new erythropoiesis-stimulating agents called for the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[12] and sodium N-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE),[13] as well as ELISA and mass spectrometric methods. All of the above-listed methods are used for the direct detection of EPO in biological fluids of athletes.

An indirect method for the detection of the abuse of erythropoiesis-stimulating agents is based on the haematological profile of the athlete biological passport (see Ref. 14).† The fundamental principle of the athlete biological passport and its haematological (blood) module is the long-term detection of EPO doping using statistical data on indirect markers. The haematological module monitors variables, such as haemoglobin, haematocrit, erythrocyte count, reticulocyte percentage and some other. This is a multidimensional approach based on longitudinal monitoring of individual performance of an athlete.

In the 2016 list of prohibited substances approved by the World Anti-Doping Agency,‡ ESAs are officially prohibited at all times, both in competition and out of competition, according to the Section S2 'Peptide hormones, growth factors and related substances'.

In the last decade, eight review articles on this subject have been published all over the world.[15–22] The present review integrates and analyzes systematically the published data on the key methods, which are currently used by WADA-accredited anti-doping laboratories around the world to detect doping with erythropoiesis-stimulating agents, and describes additional biomarkers for the illegal use of ESAs and blood doping. A distinctive feature of this review is the systematic analysis of the state of the art in methods for the detection of erythropoiesis-stimulating agents using electrophoretic and enzyme immunoassay techniques employed over the last 18 years. The advantages and limitations of the techniques are considered in detail. Particular attention is given to promising approaches and investigations that can be applied to control prohibited ESAs in the near future.

II. Structure of erythropoiesis-stimulating agents

By chemical nature, erythropoietin belongs to acidic glycoproteins. These proteins contain heterosaccharides with a small number of monosaccharide residues (bi-, tri- and tetraantenna structures), which are covalently attached to the polypeptide chain and differ in the degree of branching of N-linked carbohydrate chains. Endogenous erythropoietin has a molecular mass of $\sim$30.4 kDa[23–26] and consists of one polypeptide chain containing 166 amino acid residues and attached sugars. The molecule contains four glycosylation sites, three of which are N-linked (to asparagines Asn24, Asn38 and Asn83) and one is O-linked (to serine Ser126) (Fig. 1). Therefore, EPO is not homogeneous but is a mixture of many isoforms, which differ in the character of glycosylation and sialylation. The differences in the sialylation result in different charges of EPO isoforms, the molecules always retaining the same amino acid sequence.[23] Due to differences in the charge, EPO can be separated into isoforms, which form the so-called EPO isoform profile during isoelectric focusing. In the erythropoietin molecule, there are intramolecular disulfide bonds between the cysteine residues: Cys7–Cys161 and Cys29–Cys33. The reduction of disulfide bonds followed by carboxymethylation of free SH groups causes irreversible structural changes and the loss of biological activity of EPO. It was shown that some amino groups and carboxy groups, as well as tyrosine and tryptophan residues, are essential for biological activity of EPO.[23,27–29]

Sialic acids play an important role in the functional activity of EPO. The removal of these acids by means of sialidase gives desialylated EPO, which is unable to stimulate *in vivo* erythropoiesis. Since sialidase that induces the desialylation of glycoproteins was found in the plasma, the desialylation is the initial stage through which EPO and other plasma glycoproteins are removed from the circulation. Plasma sialidase continuously removes sialic acid residues from the glycoprotein substrate, resulting in the binding of naked galactose residues to liver cell membrane receptors. The desialylation causes a slow degradation of EPO and the loss of its biological activity.[23]

The creation of additional N-glycosylation sites in the EPO structure was the first attempt to increase the half-life of erythropoietin preparations. Darbepoetin alfa is characterized by higher degrees of glycosylation (five N-linked

† See https://wada-main-prod.s3.amazonaws.com/resources/files/wada_abp_operating_guidelines_2014_v5.0_en.pdf

‡ The World Anti-Doping Agency prohibited list, 2016. International Standards: https://wada-main-prod.s3.amazonaws.com/resources/files/wada-2016-prohibited-list-en.pdf

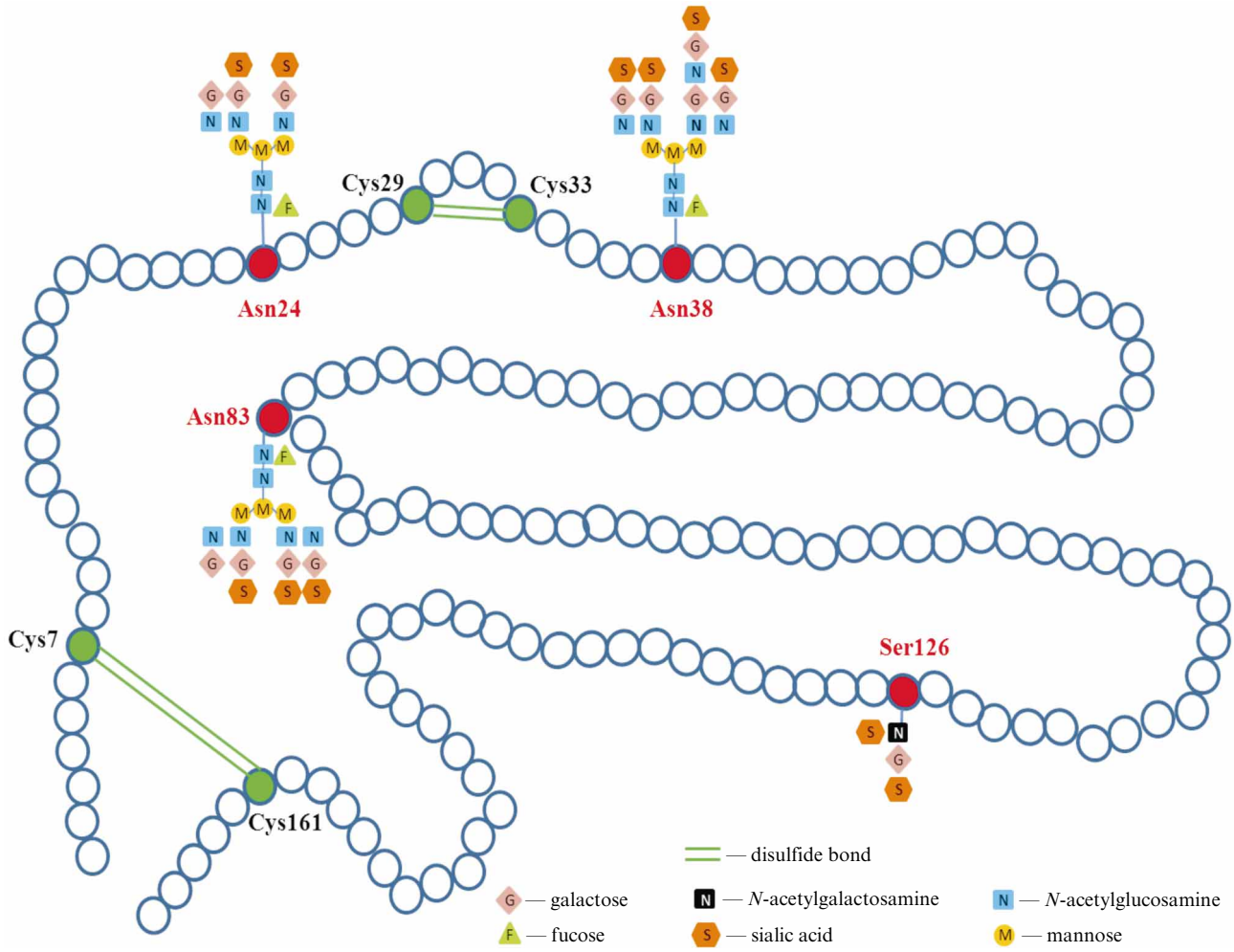


Figure 1. Structure of endogenous erythropoietin.

carbohydrate chains *versus* three chains in epoetins and endogenous erythropoietin) and sialylation and the presence of five amino acid substitutions. Figure 2 schematically displays EPO preparations and shows glycosylation sites. The higher the degree of glycosylation, the longer the half-life of epoetin. Since recombinant epoetins contain a different number of glycosylation sites and a different number of sugars, they differ in the molecular mass. The typical molecular masses estimated by SDS-PAGE for various epoetins are as follows: uEPO (urinary) and shEPO (serum), ~34 kDa; epoetins alfa (Eprex, Erypo) and beta (Neo-Recormon), ~36–38 kDa; darbepoetin alfa (NESP), 44–45 kDa; epoetin delta (Dynepo), ~36 kDa.[19]

The next step in reducing the frequency of injection of EPO preparations by increasing the half-life was the design of a new semisynthetic EPO preparation by the Swiss company Hoffmann–La Roche. Mircera (methoxy polyethylene glycol–epoetin beta), a continuous erythropoietin receptor activator, differs from human erythropoietin in that it contains an amide bridge between the N-terminal amino group (Ala1) or the ε-amino group of lysine (at the positions Lys52 and Lys45) and methoxy polyethylene glycol butanoic acid[30] (indicated by arrows in the bottom in Fig. 2). The molecular mass of Mircera is 69–78 kDa,[19] which is much higher that the renal filtration threshold (40 kDa), and, correspondingly, this drug has a longer half-life than the epoetins created earlier.[31] Polyethylene glycol

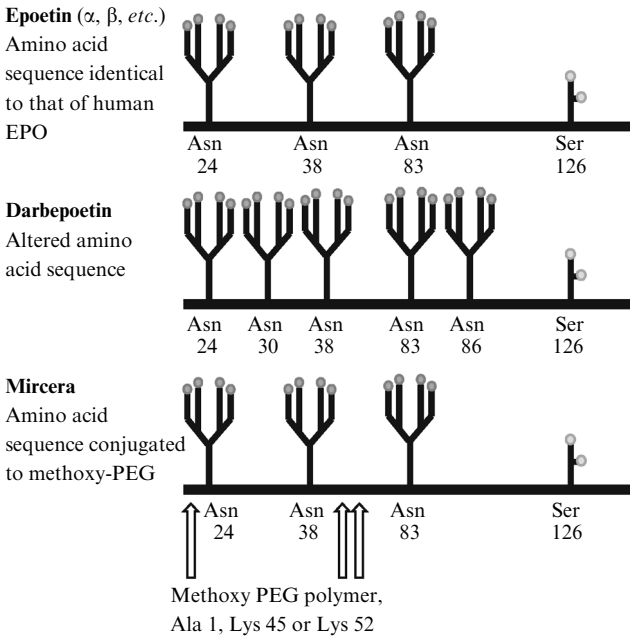


Figure 2. Schematic view of epoetin, darbepoetin and Mircera. Numerals indicate the positions of the glycosylation sites [asparagine (Asn) N-linked glycans, serine (Ser) O-linked glycan] and the PEGylation sites (for Mircera).[30]

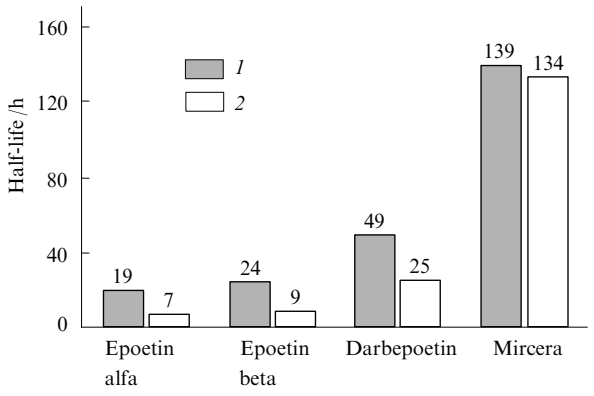


Figure 3. Half-lives of some erythropoiesis-stimulating agents compared with Mircera.[31]
(*1*) Subcutaneous administration; (*2*) intravenous administration.

(PEG) is often used as a carrier for bioorganic compounds. Owing to high hydrophilicity, a PEG molecule can bind several water molecules. This leads to the formation of a water shell around the modified PEG–protein complex, resulting in a considerable increase in its hydrodynamic radius. Due to a substantial increase in the hydrodynamic radius of the molecule, the clearance of biological agents *via* renal filtration slows down 10–100-fold depending on the PEG length. Therefore, the PEGylation extends the duration of action of proteins. Thus, a prolonged therapeutic effect is achieved after a single administration, fluctuations in the blood level of the drug, which cause adverse side effects, are avoided and the toxicity of the drug is decreased.[32] Mircera is willingly used by unfair athletes. The effect of a single injection of this drug is retained up to one month. Figure 3 presents data on the half-times for some rEPO preparations and Mircera.

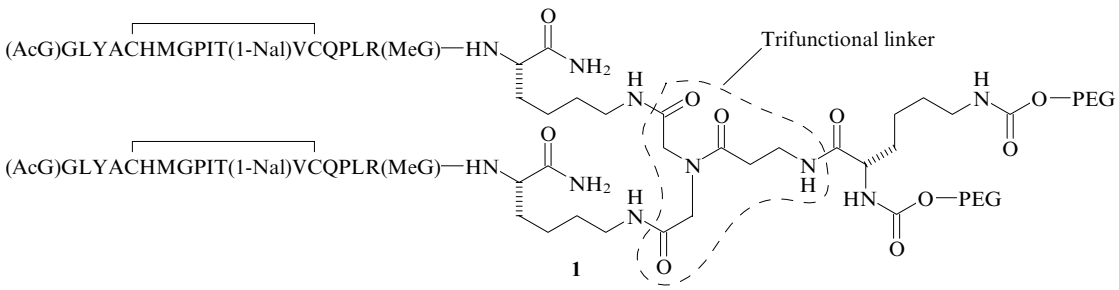
Peginesatide (**1**), a semisynthetic EPO-mimetic peptide, is also PEGylated.[10,33] This compound is a dimeric EPO-mimetic peptide (the molecular mass of each peptide is ~2.4 kDa) that contains three non-proteinogenic amino acid residues [*N*-acetylglycine (AcG), 1-naphtylalanine (1-Nal) and *N*-methylglycine (MeG, sarcosine) in positions 1, 13 and 20, respectively] and two PEG groups with a molecular mass of 20 kDa.[33] However, peginesatide was not approved for pharmaceutical use because of a high incidence of cardiovascular events and mortality in clinical trials.[4,5]

The half-life of peginesatide in the body can be elongated either through EPO dimer formation or by fusing an EPO molecule with other proteins, for example, with albumin,[34,35] human chorionic gonadotropin β-subunit (β-hCG)[36] and some other. These changes decrease the affinity of EPO for its receptor but they slow down the release of EPO from the bloodstream because of less efficient glomerular filtration.[37] The EPO-Fc hybrid protein consisting of EPO and the Fc fragment of human immunoglobulin G (IgG) has been engineered in recent years. The endocytosis of EPO-Fc mediated by the interaction of the Fc fragments with FcRn receptors,[38] which are expressed in the vessel endothelium and the lung epithelium, enables the transport of this protein after inhalation and an increase in the half-life,[39–41] thus extending the duration of therapeutic activity. The lungs were chosen as the target organ for drug delivery due to high expression of FcRn in their epithelium, as well as taking into account that the delivery through this organ has proven successful with low-molecular-mass proteins.[42] The EPO-Fc dimer consists of two EPO molecules fused to the Fc dimer by a linker, which can contain 2 to 16 amino acid residues (mainly glycine, serine, alanine and threonine).[6] The EPO-Fc monomer contains one EPO molecule attached to the dimeric Fc fragment. Based on the information obtained from one of the manufacturers,§ EPO-Fc has a molecular mass of ~140 kDa and consists of 794 amino acids: two human EPO molecules (UniProt P01588, 2×165 amino acids) and two Fc fragments of human immunoglobulin G (UniProt P01857, a part of the hinge, the CH2 and CH3 domains, 2×232 amino acids).[11] Data on clinical trials of EPO-Fc-based preparations are lacking, but these compounds are available for research studies.

In view of the above, direct methods were developed based on the electrophoretic separation of erythropoietin molecules followed by the immunological detection (Western blotting). These methods can be used to separate EPO molecules by differences in charges (IEF-PAGE) or molecular mass (SDS-PAGE, SAR-PAGE) and are currently the major methods for the detection of EPO apart from ELISA techniques and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Based on these data, recombinant erythropoietin can be distinguished from endogenous erythropoietin. Since the EPO content in urine and serum (or plasma) samples is low, the detection of this agent requires the sample preparation involving the concentration and purification of EPO from interfering proteins. Biological samples are concentrated using ultrafiltration on membranes with a pore size of 30 kDa to produce a concentrate of the protein with a molecular mass > 30 kDa in a volume of 40–50 μl from 20 ml of the urine. Erythropoietin is extracted from the concentrate by

§ The official site of the Prospec company http://www.prospecbio.com/EPO-alfa_Human_Fc_2_14

Structure 1



1

means of immunoaffinity purification, different modifications of which are described below.

III. Immunoaffinity purification of erythropoiesis-stimulating agents

In order to improve the quality of routine analysis for the detection of EPO, anti-doping laboratories employ immunoaffinity purification of erythropoietin from urine, serum and plasma.[43,44] The immunoaffinity purification is a facile and powerful tool for the sample preparation in order to enhance the signal-to-noise ratio in the detection of proteins with low concentrations, and it is applied prior to such analytical methods, as IEF-PAGE, SDS-PAGE, SAR-PAGE and LC-MS/MS.[45] This purification improves the electrophoretic separation and prevents protein bands from being smeared or wavy within lanes due to an excess of major proteins in the sample. In the WADA technical document TD2014EPO, it is strictly recommended to use one of immunoaffinity purification methods for the sample preparation in the analysis of the samples for the presence of rEPO. Below we will consider three main methods of immunoaffinity purification for anti-doping control.

The immunoaffinity purification of urine samples prior to the analysis for the presence of recombinant EPO by SDS-PAGE and SAR-PAGE is performed mainly in ELISA plates[44,46] manufactured by StemCells.[47] In this case, the immunoaffinity purification involves the dilution of 20 μl of concentrated urinary proteins in 50–80 μl of a buffer (from the kit), pH 7.4, supplemented with a nonionic surfactant in order to reduce nonspecific binding of matrix proteins followed by the incubation for 1 to 12 h in ELISA plate wells. Then the wells are washed, and EPO is eluted with various buffers. When using a 4.4% solution of the nonionic surfactant 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as an elution buffer, samples are suitable not only for SDS-PAGE and SAR-PAGE but also for IEF-PAGE.[47]

Another promising approach to the immunoaffinity purification of EPO is based on the use of an immunoaffinity sorbent[48] on an inert support, for instance, on Sepharose or magnetic particles with immobilized erythropoietin-specific antibodies. Various covalent linkers are sometimes used to prevent the washing of the sorbents away from the support. A sorbent prepared by the covalent linking of antibodies to a support can be repeatedly used for this purpose.[43,44]

In routine work, many anti-doping laboratories use disposable monolith columns available from MAIIA Diagnostics (Sweden) for the rapid purification and concentration of EPO and its analogues from urine, serum or plasma.[49] Each column is immobilized with 20–40 μg of mouse anti-EPO monoclonal antibodies. This method enables the purification of erythropoietin in one step, without employing additional methods for the concentration, from 20 ml of urine or 2 ml of serum (or plasma). In order to reduce the viscosity and dissolve protein aggregates, the samples are pre-diluted with a buffer supplemented with a surfactant and other chelating agents. Urine samples are additionally heated to cause reversible renaturation of a protein flocculent precipitate that is formed during the transport and storage predominantly from the renal Tamm–Horsfall glycoprotein.[50] The sample is passed through a support immobilized with antibodies, and the bound erythropoietin is desorbed in acidic conditions using ~50 μl of a buffer. The degree of extraction of urinary erythropoietin is ~65%, this value being dependent on the type of erythropoietin. The lowest degree of extraction was obtained with Mircera (~30% from urine samples). Studies of the effect of the purification by means of columns on the behaviour of EPO in the IEF-PAGE analysis revealed no changes.[48] Up to 24 samples can be simultaneously purified by this method provided that an appropriate vacuum device (manifold) is used.

IV. Direct methods of erythropoietin detection

At the present time, the strategy for the detection of rEPO is regulated by the WADA technical document TD2014EPO¶ 'Harmonization of analysis and reporting of erythropoiesis stimulating agents (ESAs) by electrophoretic techniques'. This document includes strict identification criteria for particular rEPO and methodologies to be applied for the initial screening or the confirmation. The technical document does not regulate the application of ELISA techniques or mass spectrometry; however, these methods can be used as additional tools to confirm the presence of a particular rEPO in a biological sample. According to the guidelines in TD2014EPO, sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis is a method of choice for the initial testing. This method reduces the number of atypical results (compared with IEF-PAGE) and has higher sensitivity in the detection of Mircera. Sodium dodecyl sulfate polyacrylamide gel electrophoresis is employed to confirm positive samples in order to detect all ESAs, except for Mircera. In the case of atypical results, isoelectric focusing polyacrylamide gel electrophoresis is used for the confirmation of the presence of darbepoetin or Mircera. All of these methods are described in more detail below.

IV.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The use of this method for the detection of recombinant erythropoietins in samples of athletes is based on the difference in the molecular masses of endogenous and exogenous EPOs.[12,51–57]

The anionic surfactant sodium dodecyl sulfate masks the charge of proteins by forming anionic SDS-EPO complexes. Disulfide bonds between cysteine residues can be broken by the addition of thiols as reducing agents, for example, D,L-dithiothreitol (DTT). This leads to a destruction of the tertiary structure of the protein and conformational changes. Amino acid chains bound to SDS form ellipsoids with identical central axes and specific charges. The applied electric field causes the SDS-EPO complexes to migrate into the polyacrylamide gel, where they are separated due to friction against the two-dimensional gel network, as well as due to the difference in the size of the polypeptide chains bound to SDS molecules. Large SDS-EPO complexes (for example, Mircera) face larger frictional forces and, correspondingly, these complexes have a lower electrophoretic mobility compared with small complexes, *i.e.*, lower-molecular-mass proteins migrate faster through the polyacryla-

¶ See https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-TD2014EPO-v1-Harmonization-of-Analysis-and-Reporting-of-ESAs-by-Electrophoretic-Techniques-EN.pdf

mide gel to the anode. In polyacrylamide gel electrophoresis in the presence of 0.1% SDS, the distance of migration of the SDS-EPO complex is a linear function of the logarithm of the molecular mass, *i.e.*, the separation is inversely proportional to the logarithm of the molecular mass of EPO.[58, 59]

Since EPO preparations contain glycosylated forms with different molecular masses,[60] the EPO bands in SDS-PAGE are broader than the bands of non-glycosylated proteins.

Prior to the application of SDS-PAGE, EPO is subjected to immunoaffinity purification from a biological material. Otherwise, the gel is overloaded with proteins, which can cause band distortions. The sample preparation involves ultrafiltration of 20 ml of urine, which is adjusted to pH 7.4. The latter is necessary in order to retain the EPO isoforms, as well as for the dissolution of the precipitate, which can contain erythropoietin. During the sample preparation, a mixture of protease inhibitors is added to the sample, resulting in the suppression of the activity of acid proteases and glucanases that are present in the urine. The resulting sample with a volume of ~20–40 μl is subjected to immunoaffinity purification, eluted and applied onto a gel. Samples of blood serum (plasma) are prepared using monolithic columns[49] manufactured by MAIIA Diagnostics. In routine diagnostic practice, anti-doping laboratories use precast 10%–12% polyacrylamide gels with the Bis-Tris working buffer (10%–12% Bis-Tris Gels). The electrophoresis is followed by electrotransfer to a polyvinylidene difluoride (PVDF) membrane. Then the membrane is blocked in milk, disulfide bonds are broken in a DTT solution and the membrane is incubated with mouse anti-human EPO primary antibodies. Immediately after washings, the membrane is visualized using secondary antibodies conjugated to horseradish peroxidase and a chemiluminescent substrate. Western single blotting is used if urine is a biological matrix to be analyzed. Western double blotting is recommended for the confirmation testing of biological samples of blood serum or plasma. For the confirmation procedure, the immunoaffinity purification prior to the application onto the gel is also a necessary stage in all electrophoretic analysis methods.

Figure 4 presents the SDS-PAGE image for some EPO preparations relative to urinary/blood serum erythropoietin (uEPO/bEPO), as reported in the TD2014EPO document. Urinary/blood serum EPO are in the third and seventh lanes. The references as a mixture of epoetin delta, NESP and CERA are shown in the fourth and eighth lanes. Various α- and β-rEPO preparations are presented from the left to right in the first, second, fifth and sixth lanes (NeoRecormon, Erypo, Beijing 4 rings and Shanpoetin, respectively) (see also the WADA document TD2014EPO).[18] The lanes of EPO preparations differ from that of endogenous EPO by the positions in the gel and in the shape and width; for example, the focused band of epoetin delta or a more diffuse band. The electrophoretic mobility of EPO preparations, as well as the band shape (width, focused or more diffuse), can be used to confirm the endogenous or exogenous origin of the substance.

The SDS-PAGE method is successfully applied to detect the doping rEPO preparation, Dynepo.[61] Due to a narrow mass distribution range of glycosylated forms of Dynepo,[62] the doping agent forms a rather clear-cut band in the gel after SDS-PAGE, which distinguishes this substance from other epoetins.[12, 18] The presence of epoetin delta can be

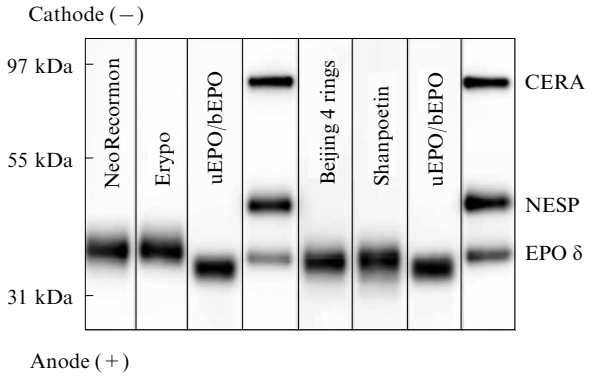


Figure 4. SDS-PAGE image showing bands characteristic of some commercially available rEPO preparations.[18]

detected by SDS-PAGE during a longer period of time compared with the IEF-PAGE method.

As the methods for the detecion of EPO, resulting in the reliable detection of large doses of EPO preparations in doping control were improved, athlets switched to microdosing.[63] The administration of large doses suppresses the production of the own hormone. Therefore, analysis for the presence of exogenous EPO clearly shows its profile. After the use of small doses of the preparation, the biological sample contains both endogenous and recombinant erythropoietin, giving rise to mixed profiles, which are rather difficult to interpret. Figure 5 presents a comparison of the mixed and non-mixed profiles of endogenous and recombinant EPOs obtained by the SDS-PAGE analysis. The mixed profile is indicated by an arrow on the right. The diffuse region above the related endogenous EPO band attests to the presence of α- and β-rEPOs. The band of endogenous EPO is shown in the central region of the lane; the rEPO epoetin delta, on the left (see the document TD2014EPO).

In order to improve the separation of protein bands in such mixed profiles, the impact of enzymatic desialylation on the urinary and serum EPO profiles was studied by SDS-PAGE.[64] It was found that the removal of sialic acid residues from various epoetins using neuraminidase reduces their molecular mass and increases the difference in the

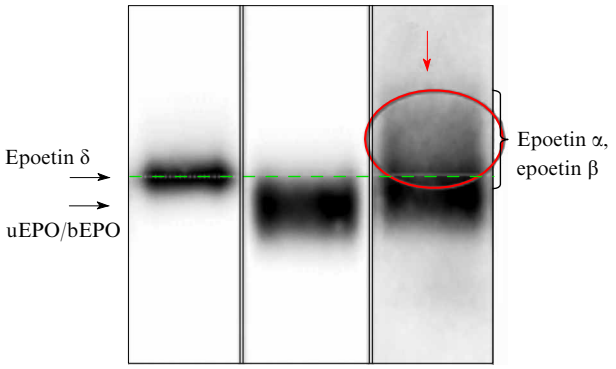


Figure 5. Identification of mixed EPO profiles.[18]
From the left to right: rEPO epoetin delta, endogenous EPO, mixed profile.

migration (*i.e.*, the distance between the centres of the bands) of recombinant and human EPOs. This approach makes it possible to avoid the band overlap, which substantially improves the detection of EPO.

Reichel reported [65] the results of deglycosylation of EPO with exoglycosidases followed by SDS-PAGE analysis and showed essential structural differences between endogenous and recombinant epoetins. The enzymes α-sialidase and β-D-galactosidase have the same effect on both endogenous and recombinant EPOs, whereas *N*-acetyl-β-D-glucosaminidase does not interact with endogenous EPO as opposed to recombinant EPO. The visualization by SDS-PAGE clearly shows that the molecular masses of endogenous EPO remain unchanged after the treatment by the first three exoglycosidases and even upon subsequent digestion with α-D-mannosidase and β-D-mannosidase. The treatment with *N*-acetyl-β-D-glucosaminidase and mannosidases leads to a substantial decrease in the molecular masses of rEPO. Both urinary and serum endogenous EPOs show this blocking effect, whereas all of the known recombinant epoetins were accessible to further degradation by mannosidases. The majority of EPO pharmaceuticals are produced in Chinese hamster ovary, baby hamster kidney (BHK) or human fibrosarcoma (HT-1080) cell lines. Since endogenous EPO is primarily produced by the kidneys, the altered behaviour might be explained by the tissue-specific glycosylation.[65]

The WADA technical document TD2014EPO describes the criteria for the assessment of the results of SDS-PAGE analysis of various recombinant erythropoietins. All results should be processed using the GASepo software package developed in the anti-doping Seibersdorf Labor GmbH (Austria).[66–68] The sample is reported as negative if the protein band visually corresponds to the endogenous EPO band and lies below the bands of rEPO preparations, which are used as the reference samples (epoetin delta, α- and β-rEPO, NESP and CERA). Even a slight shift of the protein band toward the reference rEPOs is flagged as suspicious and it triggers further investigation.

IV.2. Sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis

As mentioned above, in the WADA technical document, SAR-PAGE is recommended to be used as the initial screening for EPO in biological samples of athletes. After the development and marketing of the Mircera pharmaceutical, it was found that the SDS-PAGE method is less sensitive to Mircera compared to other epoetins due to the interaction of primary antibodies used in the Western blotting (clone AE7A5) with larger (>30 kDa) polyethylene glycol fragments in the Mircera structure. In order to address this issue, it was proposed [13, 69] that SDS in the sample and running buffers for SDS-PAGE be exchanged for sodium *N*-lauroylsarcosinate. Sarcosyl (SAR, sodium *N*-lauroylsarcosinate), like SDS, is an anionic surfactant and a methylglycine derivative but, as opposed to SDS, it virtually does not interact with polyethylene glycol fragments of Mircera and is involved in interactions only with the polypeptide chain. Therefore, Mircera gives an intense band in SAR-PAGE analysis.[13, 69] The sequences of stages in the analysis by the SAR-PAGE method is similar to that used in SDS-PAGE, except for the presence of sodium *N*-lauroylsarcosinate in the running and elution buffers instead of sodium dodecyl sulfate.

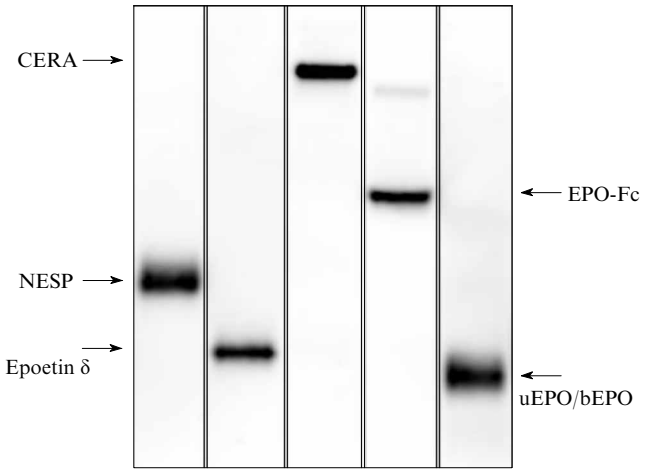


Figure 6. SAR-PAGE image showing bands characteristic of CERA, EPO-Fc, NESP, epoetin delta and endogenous EPO (see the document TD2014EPO).

As can be seen in Fig. 6, the electrophoretic mobility of Mircera, EPO-Fc, NESP and epoetin delta is lower than that of endogenous erythropoietin. The protein bands of these preparations lie above the uEPO/bEPO reference. Blood serum or plasma samples are preferable for the detection of Mircera abuse, because only a small amount of this substance is excreted with urine. The half-life of Mircera in the blood serum is ~130 h. Another advantage of the testing of the blood serum or plasma is that intense exercise has no effect on the level of endogenous serum erythropoietin.[70]

The identification criteria described in the WADA technical document TD2014EPO for the SDS-PAGE and SAR-PAGE methods are the same. Both methods perform identically except for the higher sensitivity of SAR-PAGE for Mircera.[4] Therefore, erythropoiesis-stimulating agents can be distinguished from endogenous EPOs (uEPO, bEPO) based on the characteristic shape of the protein band and the difference in the molecular mass. If the screening procedure is carried out by the SAR-PAGE method, the confirmation of the suspicious result should be carried out by means of SDS-PAGE, the preliminary immunoaffinity purification of the concentrated sample being mandatory. For the confirmation of the presence of Mircera or NESP, the IEF-PAGE method can be used as well. Once Mircera is detected, the sample preparation of serum (plasma) samples should be performed using MAIIA Diagnostics columns.

IV.3. Isoelectric focusing polyacrylamide gel electrophoresis

Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) is yet another electrophoretic method for the detection of recombinant erythropoietin in samples of athletes.[3] Isoelectric focusing of EPO in polyacrylamide gel followed by double immunoblotting and chemiluminescent detection was the first method, which made it possible to reveal recombinant erythropoietin abuse as doping.[3] The first validated anti-doping analysis using this principle was developed by Francoise Lasne and co-workers [3, 71] in 2000. The authors took into account the results of previous studies,[72–74] which showed that:

— human urinary erythropoietin contains a larger amount of acidic isoforms than recombinant EPO or endogenous serum EPO;

— this difference can be employed to detect the presence of recombinant EPO both in urine and serum.[74]

The IEF-PAGE analysis of EPO is based on the fact that, when subjected to the electric field, EPO isoforms migrate in a pH gradient. The linear pH gradient is generated in the range from 2 to 6 (pH increases from the anode to the cathode). The migration rate for different EPO isoforms is directly proportional to their electrophoretic mobility. The isoelectric focusing technique uses the isoelectric point (pI) of the protein as a zwitterion as the fractionation parameter. All molecules having the same value of the isoelectric point are concentrated in a narrow area, where pH = pI.[58,59] Different EPO isoforms migrate across the gel thus forming the lane of the sample and are focused into sharp bands.

In the initial modification of this method, only urine was used in the analysis for the presence of recombinant EPO. At the present time, IEF-PAGE is applicable for the detection of rEPO in the blood serum and plasma. The main stages of the analysis involve the sample preparation, electrophoresis in a pH gradient from 2 to 6, double immunoblotting and chemiluminescent detection of the signal. The sample preparation prior to the IEF-PAGE analysis is similar to that used in SDS-PAGE. Thus, 20 ml of urine or 2 ml of serum produce 20 μl of the retentate or 50 μl of the eluate, which is applied onto a gel. The resulting sample (~20–40 μl) is subjected to immunoaffinity purification. The eluate is applied onto a gel and subjected to electrophoresis. After the isoelectric focusing, the proteins are blotted by semi-dry electrotransfer to a PVDF membrane. In order to break disulfide bonds, the membrane is incubated in a D,L-dithiothreitol solution, blocked with milk and incubated with primary mouse anti-human EPO monoclonal antibodies. After several washings, the second blotting is performed[75,76] in order to reduce non-specific interactions of secondary antibodies with other proteins that are present in the sample. The successful application of this stage was a key factor for the development of the IEF-PAGE method, which was later approved for doping control. The resulting membrane is blocked with milk, incubated with biotinylated secondary antibodies and visualized using streptavidin conjugated to horseradish peroxidase and with a chemiluminescent substrate. Isoform profiles of erythropoietin are processed with the GASepo software package.[66–68]

Figure 7 shows an illustration of an IEF-PAGE analysis for reference standard samples of recombinant α- and β-EPOs (rEPOs), endogenous EPO NIBSC (uEPO), darbepoetin alfa (NESP) and Mircera. The isoform profiles (the character of isoform distribution) of the standard EPO samples — the α- and β-rEPO biological reference preparations (BRPs) and CERA — are observed in the basic area of the gel. They are shown in the first and fourth lanes and are identified by numbers. After isoelectric focusing, the isoform profile of the standard NESP (third lane) is observed in the acidic area, which corresponds to the lower part of the gel.[71] The rEPO isoforms in the acidic area are identified by Latin letters. The isoform profile of urinary erythropoietin is in the endogenous area in the second lane and its isoforms are indicated by Greek letters. An international reference preparation from the National Institute for Biological Standards and Control (NIBSC, UK) was used as urinary EPO.

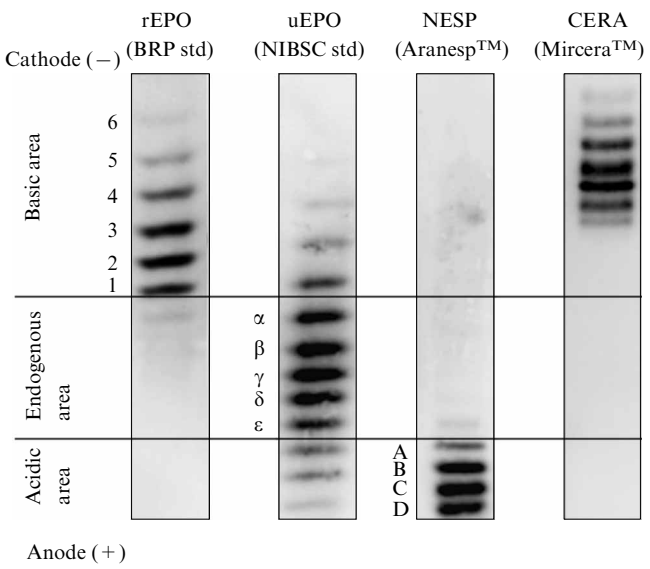


Figure 7. Image of identification windows of protein bands of reference preparations: α-, β-rEPO, NIBSC, NESP and Mircera (see the document TD2014EPO).
The basic and acidic areas are defined by the position of the protein bands corresponding to recombinant EPO preparations, namely, by the lower protein band of the biological reference (std) preparation (BRP) of the European Pharmacopeia (an equimolar mixture of α-EPO and β-EPO) based and the upper protein band of NESP; the endogenous area is located between these bands.

The WADA technical document TD2014EPO describes the criteria for evaluation of IEF-PAGE results. The result is reported as negative if the most intense bands of the EPO isoform is in the endogenous area of the gel. If the most intense bands are in the acidic or basic area of the gel and the isoform is shifted to one or another area, the result is reported as positive.

The IEF-PAGE method is a rather laborious and time-consuming as applied for the detection of EPO. The results are obtained on the third day after the beginning of the analysis. The IEF-PAGE method cannot be used to identify EPO-Fc isoforms, because the isoelectric point of this substance is outside the pH range from 2 to 6. However, experts of the Federal State Unitary Enterprise 'Antidoping Centre' (Moscow) are working on the development of the approach to the detection of hybrid EPO-Fc proteins by this method.

Besides, experts from the Moscow Antidoping Centre studied bioanalogues of EPO produced in the Russian Federation by different methods, including IEF-PAGE. Some illegal rEPO preparations were shown to be unique and have no analogues in the world.[77] The investigations revealed differences in the isoform profiles of officially approved bioanalogues of EPO and those sold on the black market. These data will be useful for improving the efficiency of doping control of athletes.

At the present time, as mentioned above, WADA does not recommend IEF-PAGE as a method for the initial screening of biological samples of athletes, and this techniques is more often used as an additional analysis in the case of suspicious samples.

IV.4. Methods based on enzyme immunoassay

Earlier, the ELISA kit produced by Microcoat Biotechnologie GmbH (Mircera ELISA kit) has been used in routine practice for the detection of Mircera. This method is an indirect competitive sandwich-type enzyme immunoassay format developed for the quantification of Mircera in the blood serum, which employs enzyme-labelled secondary antibodies and an antigen–carrier protein conjugate immobilized onto a solid support.[8] Assays were performed in microwell plates sensitized by streptavidin in order to immobilize biotinylated primary anti-EPO polyclonal antibodies (in Fig. 8, anti-EPO-bi). The sensitization was generally carried out overnight. After the washing, the sample was placed in microwells (100 μl per well) and incubated at room temperature for 2 h. Then the wells were washed, and the bound Mircera was detected using anti-PEG monoclonal antibodies (anti-PEG) labelled with digoxigenin (Dig) for 1 h. In order to detect the bound anti-PEG antibodies, the second incubation with anti-Dig-Fab conjugated with horseradish peroxidase (anti-Dig-HRP) followed by the colorimetric reaction [of the substrate with a solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) as the chromogen]. The absorption was measured at 405 nm; the reference wavelength was 490 nm. The schematic representation of this analysis is given in Fig. 8. The development and validation of this ELISA method for the detection of Mircera was described by Lamon *et al.*;[8] the limit of detection of this test is 30 pg ml^{-1}; the limit of detection of the confirmation assay is 50 pg ml^{-1}. Depending on the administration route (intravenous or subcutaneous) and individual features of a patient, Mircera can be detected within 8 days to 4 weeks.[8]

One of advantages of this method is that a large number of samples can be analyzed over a rather short period of time ($\sim$3.5 h). However, this method has a significant drawback. Thus, it gives a high percentage of false-positive results, which are later not confirmed by SAR-PAGE. The testing was usually performed on an ELISA analyzer in automatic mode, and inadequate washings between the stages of the assay or non-specific interactions of antibodies decreased the accuracy of the method. According to the recommendations of the WADA technical document TD2014EPO, this ELISA kit was not used for the detection of Mircera since 1 September 2014.

In the recent years, anti-doping laboratories have performed extensive studies on the development of commercial ELISA kits for the detection of other erythropoietins.[44, 78, 79] When creating enzyme immunoassay kits, researchers subjected samples to the preliminary treatment with enzymes[78] or employed the specific features of the elution of primary antibodies used in ELISA.[44] Recombinant human erythropoietins in samples, taken not from human sources, were detected using also species-specific differences in the primary structure of erythropoietins.[80–82] Wide *et al.*[78] showed that NESP can be differentiated from human erythropoietin by treating blood serum samples with α(2 → 3,6,8,9)-neuraminidase from *Arthrobacter ureafaciens*. Based on this principle, NESP can be detected in blood serum samples from patients 14 days after the last injection. However, this method was not approved for use in doping control.

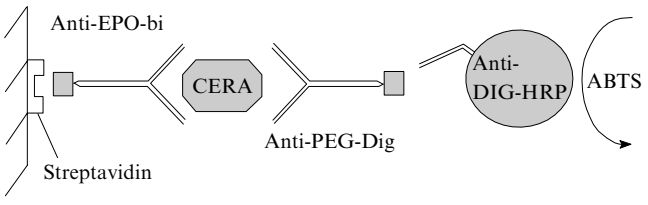


Figure 8. Schematic representation of the analysis for the detection of Mircera.

The potential of differential elution using various acidic buffers combined with commercial ELISA kits (R&D Systems) was investigated.[44] In this study, the ability of primary monoclonal antibodies from the ELISA kit (clone 9C21D11) to specifically recognize the main erythropoietin isoforms was employed. In acid wash conditions (0.7% AcOH, pH 2.8), basic isoforms were mainly washed off from the wells, whereas in alkaline wash conditions (0.4 M glycine in 6 M buffer supplemented with urea, pH 11.3) erythropoietin isoforms were not detected. This method of detection was tested by the 'added–found' procedure using blank urine samples spiked with CERA, Dynepo, BRP and NESP followed by the analysis of samples from volunteers treated with BRP. The performance characteristics of this method are similar to those of IEF-PAGE with respect to rEPO; however, the ability to differentiate NESP proved to be low. Physical exercise before the testing substantially decreases the sensitivity of the method. Problems can also arise in the case of the simultaneous presence of rEPO and NESP in the sample, which can give a false-negative result. This method is not commonly used by anti-doping laboratories.

Van Maerken *et al.*[79] developed a screening method for the detection of Mircera in blood serum using commercial erythropoietin ELISA kits (Access Epo assay; Beckman Coulter) coupled with the fractional precipitation of proteins. The method is based on the observation that the PEGylation of recombinant erythropoietin (for example, Mircera) can enhance its water-solubility, due to which serum samples containing Mircera can be specifically enriched through precipitation of blood serum proteins with PEG 6000. It was shown that the Access Epo assay kit enables the detection of Mircera within two weeks in all patients treated with doses of 50–350 μg every four weeks and, in most cases, within the third and fourth weeks. It was concluded that this test may be efficient in doping control although it has been developed for the analysis of clinical samples. However, this method is not yet approved.

Leuenberger *et al.*[9] showed that peginesatide can be identified in serum samples of athletes by a sandwich ELISA. A serum sample was added, after washing, into wells (100 μl per well) of the plate sensitized with monoclonal antibodies (clone 1A8, Affymax) and incubated for 1 h. Then the washing was repeated and the bound peginesatide was detected using biotinylated monoclonal antibodies (clone 11F9, Affymax) also for 1 h. The detection was performed using the streptavidin–horseradish peroxidase conjugate (Strept-HRP, BioSpa, Italia) followed, after washings, by the colourimetric reaction. 3,3′,5,5′-Tetramethylbenzidine (TMB) was used as the chromogen. The values for the positive control samples measured at 650 nm should fall in the range from 0.6 to 1 unit. Only then the reaction was terminated with a 1 M sulfuric acid

solution. The colour of the control and positive samples changed from blue to yellow and then the absorption was measured at 450 nm. The samples with absorption >1 were subjected to confirmation testing by the SDS-PAGE method.[9] These ELISA kits were not commercialized.

The abuse of rEPO and NESP in racehorses was detected[80] based on the differences in the amino acid sequences of human erythropoietin, NESP and horse erythropoietin. The ELISA kit (R&D Systems) was used for the screening. The confirmation was performed by IEF-PAGE. The ELISA kits were used in the same way[81] for doping control of dogs. This method was tested on approximately 6000 samples from greyhounds participating in racings. Positive results were obtained in four cases; in three cases, rEPO was detected. Human urinary EPO was once identified, which proved to be the result of a substitute of a human urinary sample for a dog sample.[82]

IV.5. Membrane assisted isoform immunoassay

Relatively recently, Lönnberg *et al.*[83–85] have developed a method that combines lectin affinity chromatography and immunochromatography in order to differentiate recombinant and endogenous erythropoietins. This method is referred to as membrane assisted isoform immunoassay.[85, 86]

The EPO WGA MAIIA strips† include the lectin zone and the capture zone (Fig. 9). After the immersion of a strip into an urine or serum sample, in the lectin zone EPO interacts with wheat germ agglutinin (WGA) specific for glycans, which contain *N*-acetylglucosamine and *N*-acetylneuraminic acid.[85] Then the bound EPO is eluted with a buffer supplemented with the competing monosaccharide *N*-acetylglucosamine (GlcNAc), resulting in that EPO isoforms are desorbed (erythropoietin is released and *N*-acetylglucosamine competitively binds to WGA) and transferred to the zone, in which the eluted erythropoietin isoforms interact with immobilized anti-EPO antibodies. The bound erythropoietin is detected using secondary anti-EPO antibodies bound to carbon black nano-strings. The assay yields a pale-grey to black signal, which is quantified using an image scanner[86, 87] and compared with the signal from rEPO preparations used as standards. The binding of wheat germ agglutinin to rEPO isoforms is stronger that the binding to endogenous EPO isoforms. The intensity of blackness is directly proportional to the concentration of the bound erythropoietin and depends on the strength of the binding of WGA to EPO. This test enabled the differentiation of epoetins, including NESP and Mircera.[88] The method is currently not in wide use in anti-doping control, and efforts are underway to improve this technique.

IV.6. Mass spectrometric analysis

In recent years, numerous attempts have been made to find differences in the structure of endogenous and recombinant erythropoietins by mass spectrometry. There are quite a few publications that report the results obtained by different research teams.[10, 89–95] The mass spectrometric determination of the ESA structure can be performed by means of the two main approaches: either at the peptide level after the controlled enzymatic hydrolysis of the amino acid sequence

† The official site of the manufacturer http://maiiadiagnostics.com/products/epo_wga_maiia/

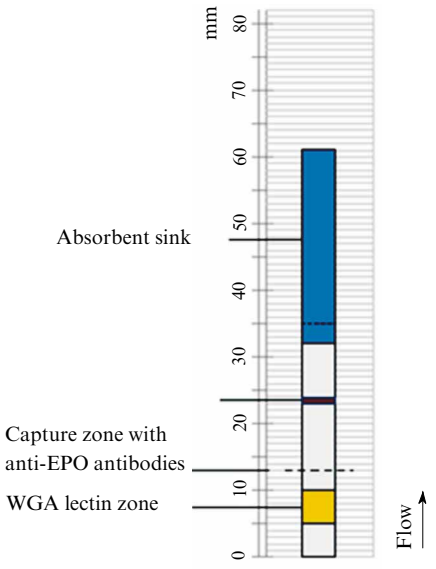


Figure 9. Schematic view of the EPO WGA MAIIA strip.[85, 86]

(digestion with trypsin or other endoproteases) or at the glycan level. The mass spectrometric detection of recombinant EPO, EPO-mimetic peptides and their chimeric analogues can be accomplished only if the EPO structure contains species-specific or artificial alterations in the amino acid sequence. In 2007, the first attempt was made to use species-specific differences between human and horse erythropoietins for the confirmation of the presence of rEPO and NESP in horse blood plasma by means of liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).[96] Erythropoietins were isolated by immunoaffinity chromatography with anti-EPO polyclonal antibodies immobilized on magnetic particles, and then the eluates containing EPO were subjected to trypsinolysis. Tryptic peptides were identified by HPLC-MS/MS based on the retention times of the analytes and the characteristic fragment ions produced by collision-induced dissociation. The preliminary deglycosylation with PNGase F followed by trypsinolysis produced the tryptic peptides 46VNFYAWK52 and 144VYSNFLR150 corresponding also to endogenous human erythropoietin. The limits of detection of the initial testing and the confirmation analysis are 0.1 and 0.2 ng ml^{-1}, respectively. Since this method is unable to distinguish between rEPO and NESP, it was modified[97] by employing some other deglycosylated tryptic peptides: 21EAENITTGCAEHCSLNENITVPDTK45 for rEPO and 77GQALLVNSSQVNETLQLHVDK97 for NESP. In this modification, the method made it possible to detect human erythropoietins in horse blood plasma; the limits of detection of the initial testing and the confirmation analysis are 0.05 (NESP) and 0.1 ng ml^{-1} (rEPO); 0.1 (NESP) and 0.2 ng ml^{-1} (rEPO), respectively. Later on, the method was adjusted[98] for the detection of NESP in human blood plasma. The achieved limits of detection of the initial testing and the confirmation analysis are 0.1 and 0.2 ng ml^{-1}, respectively. More recently,[99] the sensitivity of this technique for the detection of EPO in horse doping control was increased with the use of another tryptic peptide

digested from human erythropoietin — 54MEVGQQAVEVWQGLALLSEAVLR76 — instead of the peptides used in the works published earlier. This peptide is characteristic not only of endogenous human EPO and its analogues but also of NESP. The following limits of detection of the initial testing and the confirmation analysis were achieved: 0.1/0.05/0.3 ng ml^{-1} and 0.2/0.1/0.5 ng ml^{-1} for rEPO/NESP/Mircera, respectively.

In 2014, Okano *et al.*[95] (Anti-doping laboratory, Tokyo) developed a procedure for the detection of NESP in human urine samples. The new approach is based on the immunoaffinity purification of a human urine sample (10 ml) and hydrolysis with endoproteinase Glu-C in a bicarbonate buffer, pH 7.8, followed by the analysis of the reaction mixture by ultra-performance liquid chromatography (UPLC) on a charged surface hybrid C18 column coupled with ESI-MS/MS. The researchers found the specific peptide fragment digested from darbepoetin — alfa 90TLQLHVDKAVSGLRSLTTLLRALGAQKE117. The lower limit of detection of the initial testing was 1.2 pg ml^{-1}; the limit of detection of the confirmation analysis was 5 pg ml^{-1}. This method enables high-throughput confirmation analysis, namely 6 h for the sample preparation and an analytical run time of only 10 min per sample. This mass spectrometric technique is an innovative and powerful tool for detecting NESP for doping control testing.

Due to the presence of unusual amino acid residues in peginesatide, the mass spectrometric detection of this substance in doping control testing proved to be feasible.[10, 93, 94] The results of mass spectrometric analysis of a peptide monomer were published in 2009.[100] Later on, the research team from Cologne developed a method based on the direct enzymatic hydrolysis with subtilisin after the precipitation of proteins followed by cation-exchange solid-phase extraction. The extracts were subjected to LC-MS/MS analysis. The method was validated by *in vivo* pharmacokinetic studies of peginesatide in rat and monkey. The resulting proteotypic pentapeptide GPIT(1-Nal) was detected in all biological matrices (urine, blood and plasma) 96 h after the injection of the preparation. However, it was demonstrated that peginesatide is unstable at the storage temperature of the control sample (−18 °C). The authors supposed that peginesatide can be reduced after thawing by resuspending of the protein precipitate. However, additional studies are needed to validate the application of this method in routine practice.[10]

Another alternative approach to the determination of the origin of EPO is to find differences in the glycan structures. Earlier, it has been shown that different EPO preparations contain a different number of terminal *N*-acetylneuraminic acid (NeuAc) residues. It was found that *N*-glycolylneuraminic acid (NeuGc) is present only in recombinant erythropoietins and in insignificant amounts (for example, 2% NeuGc, 98% NeuAc) in endogenous EPO.[101, 102] Neuraminic acid residues in recombinant human erythropoietins are $\alpha(2\rightarrow3)$-linked to galactose exclusively, whereas $\alpha(2\rightarrow6)$ bonds are additionally present in uEPO.[90] The fucosylation was observed mainly at the $\alpha(1\rightarrow6)$ bond with the first core *N*-acetylglucosamine.[90, 91, 103] It was also demonstrated that the preparations contain mannose-6-phosphate[104] and sulfated *N*-acetylglucosamine residues.[105–107]

Reichel[92] proposed a mass-spectrometric method for the determination of the EPO origin using the O-acetylation of the terminal sialic acid residues as a marker. The work was focused on the O-glycopeptide (E117-R131) produced by means of trypsinolysis and the degree of O-acetylation of its sialic acids. The data were compared with the results obtained for more than 40 rEPO preparations. It was shown that the human urinary EPO O-glycopeptide (~100 IU) contains only trace amounts of mono- and disialylated glycans and contains no other O-acetylated structures, which is a substantial difference from all recombinant EPOs. However, this method has low sensitivity and is still under the development.

In 2012, Reichel and Thevis demonstrated[11] that the EPO-Fc fusion protein containing recombinant human erythropoietin and the IgG Fc fragment is detectable by the mass spectrometric nano-ESI-MS/MS method. The spectrometric identification was performed automatically with the SEQUEST search algorithm and the UniProt protein sequence database. After the identification of tryptic peptides, two EPO-Fc peptides characteristic of human EPO (EAISPPDAASAAPLR) and the CH3 domain of human immunoglobulin G (GFYPSDIAVEWESNGQPENNYK) were found. It should be noted that, according to the authors, the sequence coverage was 44%, but no further attempts were made to increase the sequence coverage and identify the primary structure of EPO-Fc.[11] This strategy needs to be further modified and improved, because attempts to find a unique peptide fragment that is formed at the junction of the erythropoietin and immunoglobulin molecules failed. The identified peptides are characteristic of both serum EPO and immunoglobulin, which are naturally present in the blood of athletes not abusing drugs and cannot be characteristic for the MS/MS detection of EPO-Fc. No other significant evidence of the presence of EPO-Fc was found. Anti-doping laboratories are pursuing studies with the aim of finding a proteotypic peptide, which would make it possible to reliably detect EPO-Fc abuse.

V. Promising approaches to the identification of erythropoiesis-stimulating agents in doping control

Apart from the above-considered methods for the detection of ESAs, which are implemented by anti-doping laboratories around the world, new approaches to the detection of rEPO and its analogues, as well as of EPO gene doping, are being developed.

Vogel *et al.*[108] employed the property of peptidic ESAs to bind to the human erythropoietin receptor. In the cited study, the erythropoietin receptor covalently immobilized on magnetic beads was proposed as a versatile method for the purification of ESAs. It was shown that this carrier can interact with endogenous human EPO, as well as with rEPO preparations, such as Mircera, EPO zeta, darbepoetin alfa and so on. After the binding, magnetic beads were washed with a phosphate buffer, and the immobilized rEPO was eluted with a 2% acetic acid solution at 37 °C for 30 min. The pH value of the eluate was adjusted to 7.0 with a 5 M aqueous ammonia solution. The eluate was concentrated to dryness and again dissolved in 30 μl of a 100 mM ammonium bicarbonate solution. A portion (15 μl) was withdrawn for the SAR-PAGE analysis, and 15 μl of trypsin was added to the remainder (100 mg per 6.5 ng of rEPO).

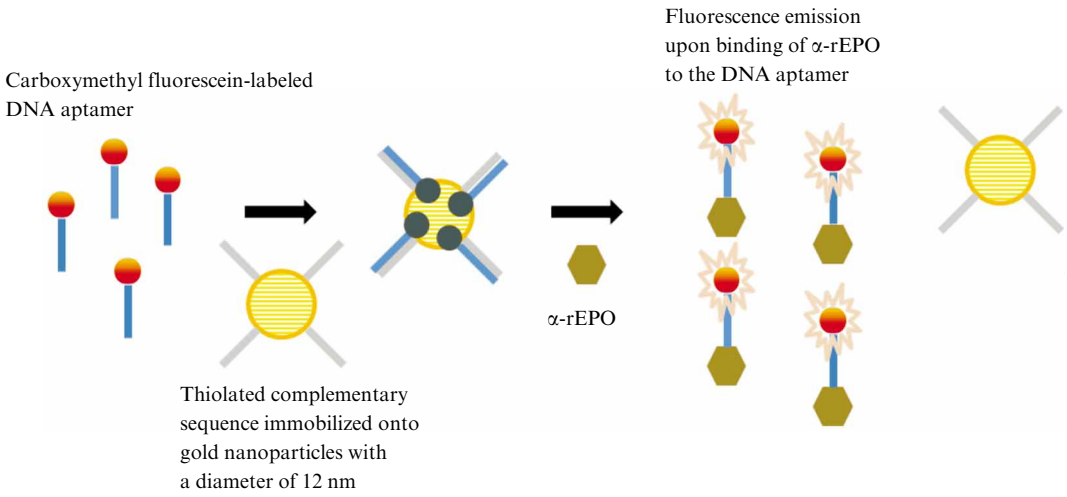


Figure 10. Detection of α-rEPO by the fluorescent labelling of gold nanoparticles.[15]

The volume was adjusted to 50 μl with an 100 mM ammonium bicarbonate solution, and the solution was subjected to trypsinolysis with the additional treatment with the enzyme PNGase F at 37 °C overnight. The reaction was terminated by adding 5 μl of glacial acetic acid, and the mixture was analyzed by LC-MS/MS. The limit of detection of EPO zeta, darbepoetin alfa and Mircera was 20, 30 and 80 ng ml^{-1}, respectively. This method of purification holds promise for the isolation of numerous ESAs regardless of their structure.

A Japanese research team revealed difference in the interaction of endogenous and recombinant urinary EPO with various lectins. Studies using glyco-chips showed that rEPOs expressed in CHO cells do not interact with lectins, whereas urinary ESAs, except for Mircera and hybrid EPO-Fc, interact with the lectins SNA (Sambucus nigra agglutinin), SSA (Soybean seedling agglutinin) and TJA-I (Trichosanthes japonica agglutinin). Based on these results, a method was proposed for the quantification of endogenous and recombinant EPO in a mixture of biological fluids of athletes. The method involves the initial purification of EPO using lectins followed by the ELISA quantification of the concentration of the bound erythropoietin.[109]

Citartan *et al.*[110] proposed the use of ultraviolet-visible-near-infrared (UV-vis-NIR) spectroscopy for the identification of rEPO in order to monitor the binding of molecular recognition elements — anti-EPO antibodies and anti-EPO DNA aptamers — to the target EPO by measuring reflectivity changes at wavelengths of 300 to 1400 nm, which can be tracked in real time. The aptamer is an artificially synthesized single-stranded DNA or RNA consisting of identical nucleotide sequences at the termini and the variable (20–30 nucleotides) central part capable of recognizing the protein target with high affinity and specificity. These artificial molecules are developed by the SELEX (systematic evolution of ligands by exponential enrichment) process. A polycarbonate platform coated with a thin gold layer (5–50 nm), on which antibodies and DNA aptamers were immobilized, was used as a reflection element. Spectrophotometric studies of molecules in the visible and UV regions allow for the determination of the type of electronic transitions and molecular structures.

In 2011, a biosensor was developed based on fluorescent labelling of gold nanoparticles.[15,111] In this method, if the target protein (EPO) is absent, an DNA aptamer conjugated to carboxymethyl fluorescein binds its complementary sequence on the surface of gold nanoparticles with a diameter of 12 nm, resulting in fluorescence quenching. In the presence of α-rEPO, the DNA aptamer is dehybridized from the sequence complementary to the aptamer and binds EPO, thus causing fluorescence emission (Fig. 10).

In the two above-cited works, the authors did not study endogenous EPO and performed experiments with rEPO; no direct evidence of the applicability of these biosensor systems for the differentiation between endogenous EPO and recombinant EPO abused by athletes was not provided.

A digital droplet polymerase chain reaction (ddPCR) protocol was developed for the identification of EPO transgenic elements with the objective of EPO gene doping detection.[112] A ddPCR duplex assay aimed at the simultaneous detection of EPO transgenic elements and insulin-like growth factor 1 (IGF-1) was created. The EPO and IGF-1 genes were produced from human genomic DNA by the polymerase chain reaction (PCR) followed by the hydrolysis with the restriction enzymes *DdeI* (*Desulfovibrio desulfuricans*) and *RsaI* (*Rhodopseudomonas sphaeroides*). Then the resulting fragments of genomic DNA were inserted into the virus vector pZac rAAV (recombinant adeno-associated virus) and cloned. The viral particles containing the inserted EPO or IGF-1 gene were injected in mice in a dose of 50 μl (10^{11} viral particles); 50 μl of phosphate buffer were injected as the control. Then transgenic mouse DNA was extracted from 100 μl of blood and hydrolyzed with the restriction enzymes *DdeI* and *RsaI*. The results obtained *in vivo* showed that the IGF-1 and EPO transgenic elements can be reliably detected within 33 days in DNA extracted from the whole blood. Digital droplet PCR provides absolute quantification and ensures excellent reproducibility.

VI. Indirect methods of erythropoietin detection. An athlete biological passport

Most of recombinant EPO preparations have a relatively short half-life. The EPO abuse can be reliably identified by direct methods within 3–4 days after the administration. Accordingly, the athlete biological passport (ABP) was developed as an indirect method of detection.‡ This passport is based on the longitudinal monitoring of changes in the individual biological markers of an athlete and predicts, for an individual, an expected range within which these values fall assuming a normal physiological condition. Within the framework of the concept of the biological passport, there is no need to directly reveal prohibited substances in doping control. It is sufficient to detect variations of the measured haematological markers in the blood of the athlete from the individual norms. The application of this method considerably extends the period of the reliable confirmation of the anti-doping rule violation due to abuse of erythropoiesis-stimulating agents.

During the sporting career, whole blood samples are collected and processed on haematology analyzers. The values of 11 markers are submitted to the Web-based WADA database ADAMS (Anti-Doping Administration and Management System). The values of the following measured biomarkers are taken into account: haematocrit (HCT), haemoglobin (HGB), red blood cell (RBC) count, reticulocyte percentage (RET%), reticulocyte count (RET#), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), immature reticulocyte fraction (IRF), red cell distribution width (standard deviation) (RDW-SD). Besides, the calculated stimulation index (OFF-score) was added to the database.[113–116] This index is calculated by the following formula:

$$\text{OFF-score} = \text{HGB} - 60(\text{RET\%})^{1/2}$$

The individual haematological profile of an athlete is automatically calculated from these data using the Bayesian statistical model.[117–119] The mathematical analysis enables the prediction of the range of values, within which the above-mentioned markers may naturally vary. The range, within which the variations in the markers are assumed to be physiologically normal, is called an individual credible interval. The currently used Bayesian analysis provides the evaluation of the individual credible interval and the prediction of the physiological range of values for subsequent measurements. The values of the markers that fall outside the individual credible interval are analyzed by an expert group, who can determine anti-doping rule violations and impose relevant sanctions. The mandatory requirements to the blood sample collection, transport and testing and to the interpretation of the results are clearly defined and described in detail in the WADA technical document.

Investigations aimed at improving the biological passport are ongoing, and researchers are finding additional biomarkers for the reliable detection of ESA abuse or blood doping. Lainé *et al.*[120] proposed the serum hepcidin level as a marker for the detection of erythropoietin abuse as doping. Hepcidin regulates the bioavailability of iron in plasma and then the bioavailability of iron for erythropoiesis. The goal of the investigation was to assess the effect of the single intravenous administration of rEPO on the serum hepcidin level. For this purpose, a single injection of rEPO in a dose of 50 IU kg^{-1} was administered in 14 healthy volunteers. The serum hepcidin level was quantified by competitive ELISA, and the parameters of iron were evaluated during 24 h after the injection. It was shown that the serum hepcidin level substantially increased 4 h after the injection of rEPO compared to the injection of placebo, whereas the iron level and transferrin saturation greatly decreased 12 h after the injection compared to the injection of placebo. The serum hepcidin levels estimated 12 and 24 h after the single administration of rEPO were also lower than those in volunteers administered with placebo. Therefore, the intravenous administration of rEPO causes an early and short-lasting increase in the serum hepcidin level, which, in turn, leads to a short-lasting decrease in the iron bioavailability.

Leuenberger *et al.*[121] investigated whether blood-circulating miRNAs can serve as biomarkers for the identification of autologous blood transfusion, which is a major doping technique that is still undetectable by direct methods. Small extracellular non-coding RNAs (microRNAs, miRs) measured in blood plasma are specific and sensitive markers of physiological processes and diseases. Plasma microRNA levels were analyzed by means of quantitative real-time PCR. Plasma samples were taken before and at different time points after autologous blood transfusion in 10 healthy volunteers. Other markers of erythropoiesis were also determined in the serum of these volunteers. The results demonstrated considerable changes in the expression level of circulating microRNAs. Markers for 10 microRNAs were upregulated in the samples after autologous blood transfusion compared to control samples. A 3.9-, 4.0- and 3.0-fold increase was observed for miR-30b, miR-30c and miR-26b, respectively. The EPO concentration decreased after blood reinfusion. A combination of EPO and miRNA concentration measurements in a mathematical model enhanced the efficiency of autologous blood transfusion detection. It was shown for the first time that microRNAs can be used as new blood-based biomarkers for the detection of autologous blood transfusion.

Leuenberger *et al.*[122] proposed a facile and efficient method for the detection of autologous blood transfusion based on the measurement of iron in serum samples in the presence of ethylenediaminetetraacetic acid (EDTA). The results demonstrated an increase in the plasma iron level up to 25-fold 6 h after blood reinfusion. This parameter remained elevated 10-fold one day after the procedure. The iron concentration was evaluated by the colourimetric method based on the absorbance of an iron–Ferene complex at 600 nm. The chemical reactions yielding this complex are as follows:

$$Fe^{3+}\text{-transferrin} \longrightarrow Fe^{3+} + \text{transferrin}$$

$$2\,Fe^{3+} + \text{ascorbic acid} \longrightarrow$$

$$\longrightarrow 2\,Fe^{2+} + \text{dehydroascorbic acid} + 2\,H^{+}$$

$$Fe^{2+} + 3\,\text{Ferene®} \longrightarrow Fe^{2+}\text{–Ferene®}\ \text{(complex)}$$

‡ See https://wada-main-prod.s3.amazonaws.com/resources/files/wada_abp_operating_guidelines_2014_v5.0_en.pdf

In an acid environment, iron (Fe^{3+}) is released from transferrin. In the presence of ascorbic acid, Fe^{3+} is reduced to Fe^{2+} and forms a complex with 5,5′-[3-(2-pyridyl)-1,2,4-triazine-5,6-diyl]-bis(furan-2-sulfonic acid) disodium salt (Ferene, Ferene®), the abundance of which can be estimated by colorimetry, thus quantifying iron in the sample.[122] The method uses a sample volume of 20 μl; the duration of the procedure is ~7 min.

VII. Conclusion

Extensive research on the design of ESAs has been performed since 1989, when recombinant EPO alpha was approved for the first time for clinical use. This triggers the development of new methods for the detection of erythropoiesis-stimulating agents having a history of use as blood doping agents and stimulates the adaptation of available methods to ever-changing conditions. The use of the SDS-PAGE and SAR-PAGE methods for the screening and confirmation analysis substantially enhanced the efficiency of EPO detection. The application of these methods using urine and blood samples has proven particularly successful for the detection of abuse of the rEPO preparations Dynepo, NESP, Mircera and mixtures of endogenous and exogenous EPO. Since SAR-PAGE assay exhibits enhanced sensitivity for Mircera, this method has advantages over SDS-PAGE as applied to the analysis of blood serum and plasma samples. The development of the ELISA technique for CERA provided an approach to the screening for Mircera abuse. The procedure, referred to as EPO WGA MAIIA, is yet another innovative technology, which is currently under validation as a tool for the screening for various EPOs. The mass spectrometric method was proposed for the direct detection of peginesatide. Approaches are being developed for the detection of EPO-Fc in human blood plasma and for the assessment of the degree of O-acetylation of terminal sialic acids in glycans as a possible mass spectrometric marker for differentiation between recombinant and endogenous EPO. In view of the fact that erythropoietin has a short half-life in serum, indirect methods were devised for the identification of this substance. These methods are included in the Athlete Biological Passport (ABP) programme (haematological module). Due to the assessment of the range of individual norms of an athlete, which are calculated using Bayesian statistical analysis implemented in ABP, the testing is more efficient and targeted. Since ABP is an 'open' system, new achievements in doping control of ESAs and alternative methods, for example, transcriptomics, proteomics and metabolomics methods, can be included in the passport and new markers can be added, which would provide more accurate assessment of the results. New approaches for the detection of both erythropoiesis-stimulating agents and EPO gene doping are being elaborated.

The following validated techniques are employed by the Russian Antidoping Centre: immunoaffinity purification using MAIIA columns and ELISA plates (Stemcells), IEF-PAGE, SDS-PAGE and SAR-PAGE methods, the haematological profile of the Athlete Biological Passport. These tools were used in doping control at the XXII Olympic Winter Games and XI Paralympic Winter Games is Sochi. The mass spectrometric procedure for the detection of EPO-Fc is under development.

The doping in sports continues to be and will remain a challenge due to the design of novel pharmaceutical products and improvement of their pharmacological properties. The latest trends in the development of hybrid molecules and PEGylation led to a substantial increase in the half-life of the preparations without considerable loss of biological activity. Athletes use these achievements to enhance their performance, an activity known as doping. This poses serious health risks and violates fundamental principles and spirit of the Olympic movement. Biosimilar analogues of epoetins flooded the black market and the fight against doping is an ever-growing challenge. Nevertheless, the challenges to detecting erythropoiesis-stimulating agents and other substances included in the WADA prohibited list problems will be tackled and new tools for fighting against doping in sport will be devised.

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