Biosensors and Bioelectronics 63 (2015) 86–98

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Monitoring recombinant human erythropoietin abuse among athletes

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ARTICLE INFO

Article history:
Received 7 April 2014
Received in revised form
2 June 2014
Accepted 27 June 2014
Available online 8 July 2014

Keywords:
rHuEPO
Athletes
Doping
Antibody
Aptamer

ABSTRACT

The illegal administration of recombinant human erythropoietin (rHuEPO) among athletes is largely preferred over blood doping to enhance stamina. The advent of recombinant DNA technology allowed the expression of EPO-encoding genes in several eukaryotic hosts to produce rHuEPO, and today these performance-enhancing drugs are readily available. As a mimetic of endogenous EPO (eEPO), rHuEPO augments the oxygen carrying capacity of blood. Thus, monitoring the illicit use of rHuEPO among athletes is crucial in ensuring an even playing field and maintaining the welfare of athletes. A number of rHuEPO detection methods currently exist, including measurement of hematologic parameters, gene-based detection methods, glycomics, use of peptide markers, electrophoresis, isoelectric focusing (IEF)-double immunoblotting, aptamer/antibody-based methods, and lateral flow tests. This review gleans these different strategies and highlights the leading molecular recognition elements that have potential roles in rHuEPO doping detection.

Contents

1. Introduction

The International Olympic Committee (IOC) has banned the administration of recombinant human erythropoietin (rHuEPO), which is preferred over blood doping to enhance performance, in 1987. Although blood doping increases the oxygen carrying capacity of the blood (Lippi and Guidi, 2000), it poses problems such as allergic reactions or hemolytic crisis; thus, athletes switched to rHuEPO. The illegal use of rHuEPO has becomes rampant due to advances in recombinant DNA technology and protein expression that enabled mass production of the substance. rHuEPO is a mimetic of EPO, which is a glycoprotein hormone and the important erythropoietic growth factor responsible for erythroid differentiation, survival, and proliferation (Fisher, 2003).

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http://dx.doi.org/10.1016/j.bios.2014.06.068
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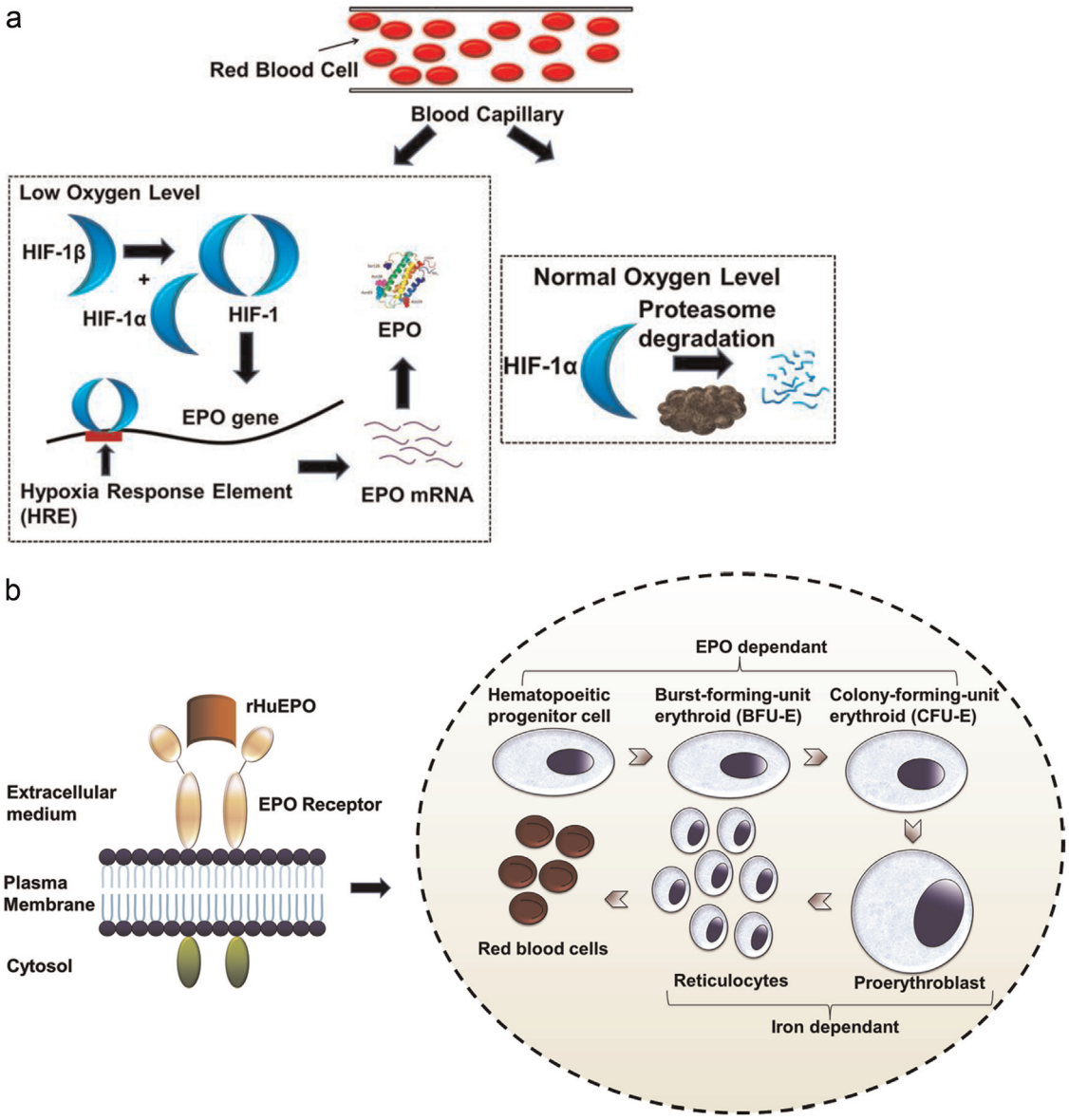

Fig. 1. (a) The level of oxygen affects the synthesis of the HIF-1α subunit, which forms a heterodimer with HIF-1β to generate transcription activator hypoxia inducible factor-1 (HIF-1), which in turn regulates EPO synthesis (Jiang et al., 1996). At the normal oxygen level, HIF-1α has a very short half-life due to its degradation by the proteosome (Huang et al., 1998; Salceda and Caro, 1997). However, under hypoxia (i.e., low oxygen level), synthesis of HIF-1α increases and dimerization with HIF-1β occurs to form HIF-1. The resulting dimer of HIF-1 binds to the hypoxia response element, which is located upstream of the EPO gene in kidney and downstream of the gene in liver (Kochling et al., 1998). This increases the transcription rate of the EPO mRNA that leads to the rise of the EPO level (Ivan et al., 2001; Jaakkola et al., 2001; Kochling, et al., 1998). (b) rHuEPO, a mimetic of EPO, binds to the EPO-receptor and increases the production of RBCs, thereby augmenting the oxygen carrying capacity of blood.

This glycoprotein is encoded by a gene located on chromosome 7, and the majority of EPO (90%) is produced in the kidney (Moore and Bellomo, 2011). EPO is initially synthesized as a polypeptide containing 193 amino acids, of which the first 27 amino acids constitute the signal peptide. Before excretion, these terminal amino acids are removed, resulting in 166 amino acid polypeptide. Oligosaccharide side chains are added at the N-glycosylation sites of the amino acid asparagine at positions 24, 38, and 83. Similar glycosylation also takes place at the amino acid serine located at position 126 (Narhi et al., 1991). These oligosaccharide side chains are required for the in vivo activity of the EPO, as they prevent fast degradation of the EPO in the liver before it reaches the target site (Jelkmann, 2008).

Many factors activate the expression of the EPO gene. The main stimulating factor is tissue hypoxia, a phenomenon whereby the oxygen capacity in the blood and the artery is reduced (Maiese et al., 2004). A low level of oxygen promotes the synthesis of HIF-1α, which dimerizes with HIF-1β to form HIF-1. This dimer binds to the hypoxia response element in the EPO gene and elevates the transcription rate of EPO mRNA, leading to the production of more EPO (Fig. 1a). The oxygen carrying capacity of the blood to the muscles is the major obstacle for performing physical activity for extended periods of time. During exercise, oxygen is very quickly consumed, which greatly limits muscular function. The administration of rHuEPO augments athletic performance by increasing the number of erythrocytes/red blood cells (RBCs), which also results in a dramatic increase of oxygen uptake (VO_{2max}) and ventilatory threshold (VT) (Audran et al., 1999; Rivier and Saugy, 1999) (Fig. 1b).

2. Recombinant human EPO

The first recombinant human EPO (rHuEPO) produced was epoetin alpha (Ashenden et al., 2012; Jelkmann, 2008). Other

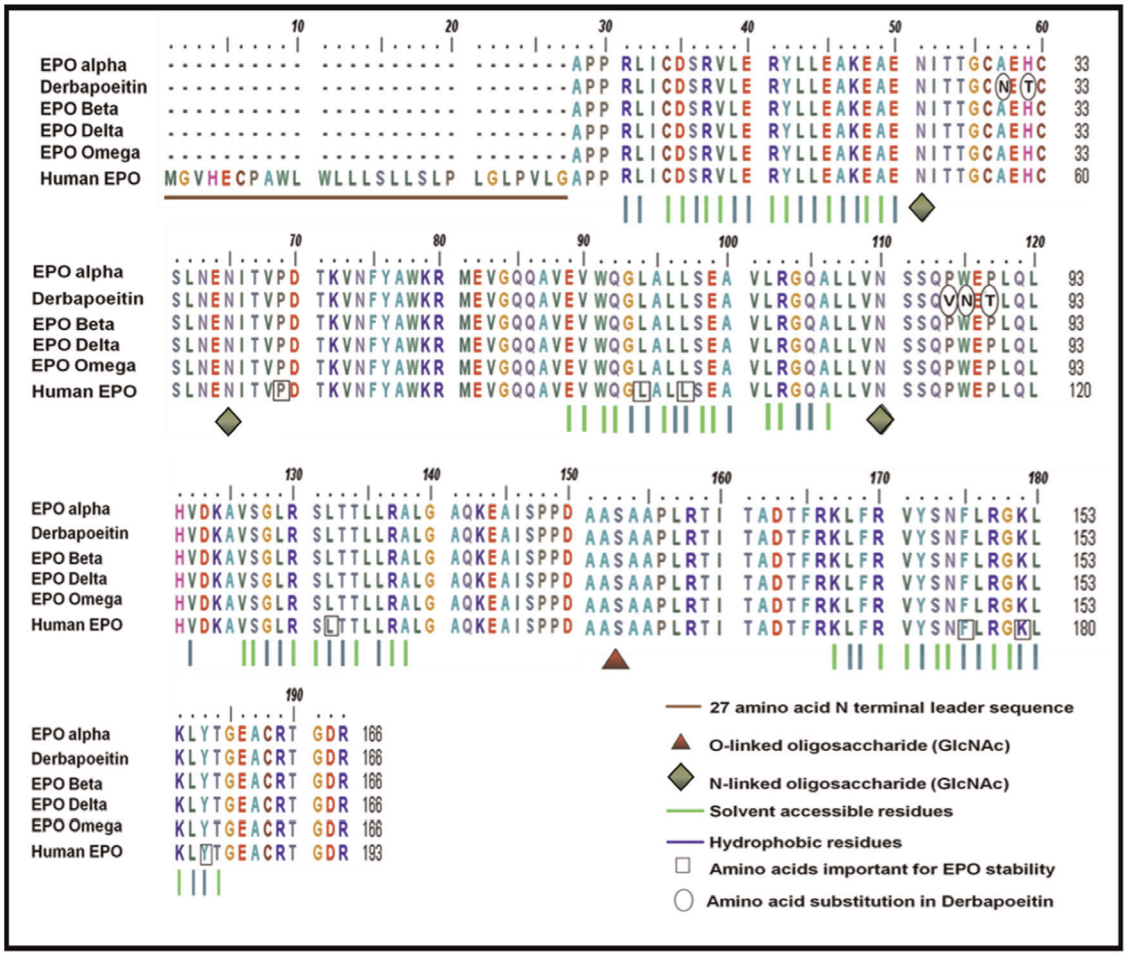

Fig. 2. Diagram showing the positions of amino acids and oligosaccharide side chains of eEPO (endogenous human EPO) and different types of rHuEPO. The leader sequence is removed from the eEPO shortly before excretion into the bloodstream. The amino acid compositions of the eEPO and all of the other rHuEPOs are the same (except for darbepoetin, which differs by five amino acids).

rHuEPOs include EPO beta, which was marketed under the names Recormon and Epogin (Storring et al., 1998), epoetin omega (branded as EPomax) (Pascual et al., 2004), epoetin delta (Deicher and Horl, 2006), and darbepoetin alpha (Aranesp and Nespo) (Egrie and Browne, 2001). The availability of these rHuEPOs (Fig. 2) has tremendously enhanced the lives of patients with chronic kidney disease, which is the key cause of anemia due to the inadequate production of EPO in the kidney (Can et al., 2013; Hattori et al., 2013). However, serious problems can arise with administration of rHuEPO. The side effects include hypertension, headaches, and increased frequency of thrombotic events due to the EPO-induced rise of hematocrit and blood thickening (Locatelli and Del Vecchio, 2003). Large doses of rHuEPO also can result in death (Lappin et al., 2002). Thus, the International Olympics Committee (IOC) decided to include rHuEPO in the "List of prohibited substances." Following this, the World Anti-Doping Agency (WADA) was established with the mission to "promote, coordinate and monitor the fight against doping in sport in all its forms". However, scandals involving illegal use of rHuEPO, such as Operación Puerto in 2006, continue to occur despite the banning of this substance. Thus, the detection of rHuEPO among athletes has become an important goal to maintain the welfare of athletes and to ensure an even playing field for all athletes. This review provides an overview of the different strategies available to detect rHuEPO among athletes and also on the leading molecular recognition elements that play a huge role in rHuEPO doping detection.

3. Tracking the hematologic parameters

Since the key problem of direct detection is the structural similarity of both the eEPO and rHuEPO, indirect detection of rHuEPO is preferred. Studies have shown that there is a relationship between EPO, iron level and erythropoietic response to anemia. These hematologic parameters-based measurements can be an indirect method of detection in which markers of the EPO level are measured rather than directly detecting the presence of the rHuEPO. This strategy is useful to detect the uptake of all types of erythropoetic stimulating agents even after more than a week of administration. One strategy is to use macrocytic hypochromatic erythrocytes as the marker for the uptake of rHuEPO. The hemoglobin concentration of these erythrocytes is <28 pg, whereas the volume is >128 fL (Casoni et al., 1993). Although this test is considered rapid and cost-effective, its sensitivity is poor, as up to 50% of the rHuEPO is undetectable when the cut-off value of 0.6% is used. Other drawback is that the individuals that have administered rHuEPO have low level of hemoglobin, which are indistinguishable from individuals with iron deficiency anemia (Macdougall et al., 1992). Moreover, before relying on this measurement as a doping control method, the analysis should involve more athletes varying in race, sport, and gender to obtain a reliable cut-off value.

Another detection strategy based on hematologic parameters is the measurement of soluble transferrin receptor (sTfR). sTfR circulates in the plasma and is produced by the transferrin

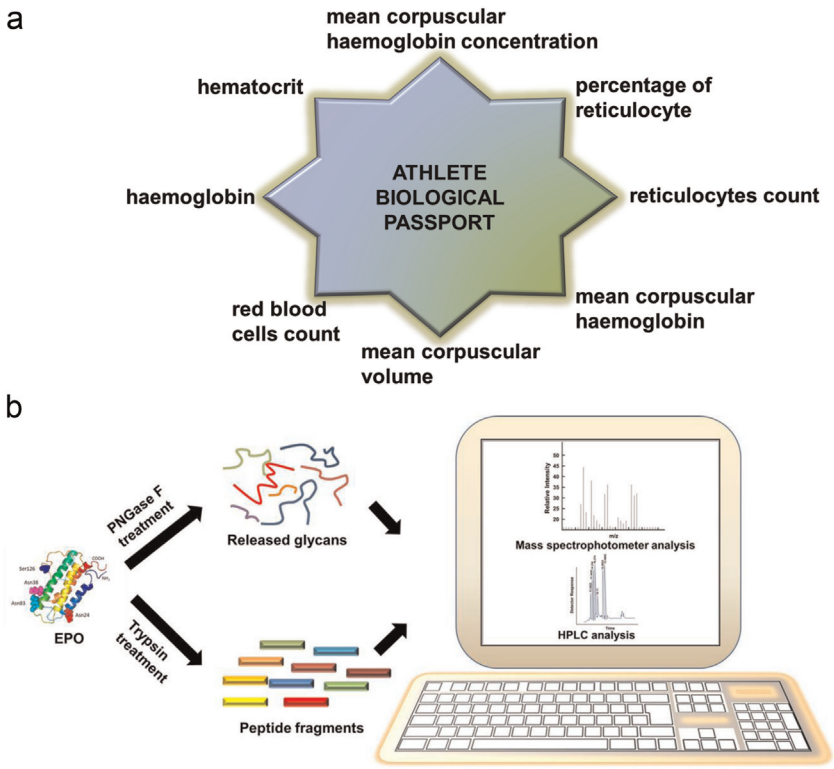

Fig. 3. (a) The athlete biological passport (ABP) consists of hematocrit, hemoglobin, RBC count, percentage of reticulocytes, reticulocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. An athlete who exceeds any of these individual values is assumed to have been using rHuEPO. (b) Glycomic study. The EPO species are treated with enzymes such as PNGase F to release N-linked glycans. These glycans are subjected to HPLC/MS analysis, which produces distinct profiles for both eEPO and rHuEPO. The analysis of these profiles enables the discrimination of eEPO from rHuEPO. In peptide-based detection, glycan digestion is omitted and the EPO species are treated with trypsin to produce peptide fragments for HPLC/MS analysis.

receptor (TfR), which allows iron to enter cells. Because the sTfR level is inversely proportional to the iron level, it can be a useful marker of the iron level and thus act as an indicator of erythropoietic activity due to rHuEPO uptake (Khatami et al., 2013). sTfR released from erythroid progenitors can be quantified by enzyme linked immunosorbent assay (ELISA) (Abellan et al., 2004). However, sTfR-based measurement can be biased because receptor levels vary with the level of iron in the human body (Bressolle et al., 1997). The level of sTfR is also elevated in individuals with anemia, those living in higher altitude and individuals with higher erythropoiesis activity, which can interfere with the interpretation of the result. The sTfR-based technique was modified to include measurement of the ferritin level, thus giving rise to sTfR/ferritin ratio-based measurement. However, additional uptake of iron can skew this ratio. Moreover, exercise changes the amount of hemoglobin present, thus affecting the value of the sTfR/ferritin ratio. This measurement was further modified to enhance its accuracy by including the RBC concentration in the blood (i.e., the hemoconcentration), in which the measurement is influenced by both the supplementation of iron and exercise (Birkeland et al., 2000).

Measuring the hematocrit, which is the percentage of RBCs in the total blood volume, is another indirect measurement technique (Saris et al., 1998). The normal threshold value of the hematocrit for males and females is 50% and 47%, respectively, and exceeding these values is indicative of rHuEPO uptake. However, the hematocrit value may vary depending on plasma volume changes, fluid loss, or conditions such as genetically determined polycythemias and iron metabolism. Furthermore, hematocrit level is also influenced by factors such as gender, age, body weight, and blood volume. Manual and automated hematocrit measurements give different values. For example, when using hematocrit centrifuge (manual), there is a false rise of the mean cellular volume, which can result in under-representation of the hematocrit value.

The drawbacks associated with each of the methods (single-parameters) described above, such as lack of specificity and sensitivity, prompted researchers to amalgamate all of these hematological parameters into a single set of measurements for discriminating between drug abusing athletes and normal athletes (multiple parameter). In this improved detection strategy, a series of hematologic parameters were combined to develop a system known as the athlete blood passport (ABP) (WADA, 2012). The ABP is a collection of the hematological parameters of an athlete, and it includes heterogeneous factors unique to the individual as the individual reference (Fig. 3a). The parameters that define the ABP are hematocrit, hemoglobin, RBC count, percentage of reticulocytes, reticulocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Based on the individual limits of the parameters, breaching of these limits is an indication of rHuEPO doping (Schumacher et al., 2012). To further fine-tune the application of ABP, Mancini et al. (2013) demonstrated that new biomarkers (dihydrotestosterone and insulin-like growth factor-1 in peripheral blood lymphocytes) can be included in the ABP (Mancini et al., 2013).

The most important factor that must be considered whether it is a single parameter or multiple parameter-based measurements is the standardization of the method to increase the precision of the result. This standardization includes contemplation of the biological, analytical and pre-analytical variabilities that can alter the data obtained from these hematologic parameters-based measurements. As an example, analytical variability which refers to imprecision and errors of the method can be reduced by thorough internal quality control methods and calibration of the instrument so that the result will not vary significantly if tested in

different laboratories for the same sample. The type of evaluation adopted also plays a pivotal role, whereby the hematological data obtained must be subjected to two types of evaluation known as transversal and longitudinal evaluation before the interpretation is finalized. Transversal evaluation refers to the comparison of the data with the cut-off value of the population while the more effective longitudinal evaluation involves comparing the data with the earlier/historical data of the same individual. However, one major caveat of the hematological parameter-based measurement that is difficult to be circumvented is as reported by Ashenden et al. (2011), whereby they have found out that administration of a very low amount of rHuEPO can result in no changes of the hematological parameters of the ABP. This limitation prompts researchers to look for alternative markers of rHuEPO uptake, which probably can give more pronounced change, such as changes in gene expression levels.

4. Gene expression pattern: the biomarker of rHuEPO abuse

Change in gene expression is often used as an indirect indicator or a gauge of any biological condition, such as disease. Similarly, the uptake of performance enhancing drugs affects the expression patterns of certain genes (Mitchell et al., 2009). As a proof-of-concept, Varlet-Marie et al. (2009) analyzed the blood transcriptome of humans before, during, and after the administration of rHuEPO. For a period of at least one week, five genes were found to be down-regulated slightly after rHuEPO administration. The direct monitoring of the EPO and EPO-receptor mRNA level is also possible, as the level of these transcripts can also be altered by the EPO administration. Other genes regulated by the EPO and EPO-receptor interaction might also undergo differential expression following the administration of EPO.

The major challenge to this approach to detecting the presence of rHuEPO is inter-individual variation in gene expression and how to determine the level of gene expression that is suggestive of drug use. One step taken to address these problems is to adopt Bayesian statistics, which rely on the reference interval obtained from a large population to adjust the inter-individual variation (Sottas et al., 2011). Other factors that can influence inter-individual gene variation include ethnicity (Storey et al., 2007), age, gender (Eady et al., 2005), health status (Sonna et al., 2004), mode of exercise (Buttner et al., 2007), medication (Lee et al., 2010a), nutrition (Bouwens et al., 2009) and natural stimulants (van Leeuwen et al., 2005). Moreover, the differential expression of mRNA between individuals that have illegally used rHuEPO and normal individuals is also less significant, which is also sometimes influenced by technical variation. Thus, an extremely large and diverse number of blood samples are required to correct for this variation. The problem of low abundance associated with the gene can also be alleviated by using deep-sequencing analysis. This analysis is able to detect low abundance transcripts owing to its sensitivity (Lee et al., 2010b). This augmentation of the sensitivity was achieved by enrichment by oligo (dT) selection prior to transcriptome analysis in the case of mRNA that has poly(A) tail at the 3′-end (Li et al., 2009; Wilhelm et al., 2008). Apart from mRNA, deep sequencing can also be used as the tool to identify mRNA that is lack of poly(A) tail or having short poly(A) tail at the 3′-end, which are up-regulated following the uptake of rHuEPO. Yang et al. (2011) have identified mRNA that do not contain the classical long poly(A) tails, which are overrepresented in specific functions. The high capacity and comparably low cost of modern deep-sequencing analysis suggest that identification of novel candidates that are up-regulated following rHuEPO uptake is possible.

An alternative approach to typical gene expression analysis involves the use of microRNA (miRNA), which refers to short non-coding RNA that mediates post-transcriptional regulation, as a potential biomarker for cancer or other diseases or for drug uptake (Ben-Hamo and Efroni, 2013; Gyparaki et al., 2013). To identify specific markers influenced by drug use, Neuberger et al. (2012) analyzed differential miRNA expression after drug administration. In another study, Serial Analysis of Gene Expression (SAGE) was used to evaluate seven thoroughbreds that were given rHuEPO-alpha. A total of 71,440 mRNA signatures were observed, 49 of which were found to be differentially expressed based on real-time PCR analysis. These identified genes exhibited inter-individual variation with strong markers (suggestive of rHuEPO uptake) that can last up to 60 days from the day of administration (Bailly-Chouriberry et al., 2010). These up-regulated miRNAs can be potential diagnostics targets for PCR or Real-time PCR-based analysis for the detection of rHuEPO administration. Genomic DNA extraction of the individual can be carried out followed by PCR analysis using the primers designed against the specific miRNA gene up-regulated following rHuEPO uptake. However, the main problem in monitoring drug uptake based on gene expression is the inter-individual variation, which can be averted by direct detection of rHuEPO or its component (peptide/glycan).

5. Glycan and peptide markers: the “fingerprinting assay”

Researchers have found that glycans on the cell surface can mediate interaction with other cells (Dwek and Brooks, 2004; Fuster and Esko, 2005). Glycomics refers to the study of the carbohydrate micro-heterogeneity of the glycans, which can differ by several orders of magnitude due to the diversity of the glycoconjugate complex controlled by the biosynthetic reactions taking place in the Golgi apparatus and endoplasmic reticulum (Aoki-Kinoshita et al., 2013; Wang et al., 2013). Differences in the glycan microheterogeneity between rHuEPO and endogenous EPO (eEPO) suggest that glycan-based analysis of these biomolecules might be useful for discriminating between EPO species (Belalcazar et al., 2006). If the amount of sample is very limited for derivatization, analysis of the native glycans can be done. Certain glycosylation sites can be targeted for differentiation of the eEPO and rHuEPO if the amount of sample is sufficient for derivatization. For example, in one study, (Skibeli et al., 2001) treated eEPO and rHuEPO with PNGase F, which removed the N-linked oligosaccharides. The removed glycans (N-linked oligosaccharides) were subjected to purification by solid-phase extraction and labeled with 2-aminobenzamide. High performance liquid chromatography (HPLC) and anion exchange chromatography were used to separate the oligosaccharides. Elution profiles of the oligosaccharides provided information about the EPOs. For example, the elution profile showed that tetra-sialylated glycan was absent in the eEPO's N-linked oligosaccharides, thereby distinguishing it from rHuEPO (Fig. 3b). Thus, analysis of the elution profile can reflect the illegal use of rHuEPO and guarantee a clear-cut result. For the analysis of the N-linked glycans, solid-phase permethylation procedure can also be used (Kang et al., 2005). However, the addition of chemical agents for stabilization of the urine sample prior to analysis is vital (Tsivou et al., 2011). Chromatography is often used with mass spectrometry (MS) analysis as another method of detecting the presence of rHuEPO in urine. In an improved methodology, two-dimensional chromatography system has been developed to map the N-linked glycans followed by MALDI TOF-TOF structural analysis (Hato et al., 2006).

MS is also applied in glycomics to provide mass and abundance profiles of the glycans present. Three different methods of ionization are available: fast atom bombardment ionization (FAB) (Zaia, 2004), matrix-assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988), and electrospray ionization (ESI) (Gyenge-

Szabo et al., 2013). Sasaki et al. (1988) have used (FAB)-MS in combination with the HPLC method to distinguish between eEPO and rHuEPO. In this study, the intact protein was digested by proteinase and separated using reverse phase HPLC followed by treatment with PNGase F to remove N-linked glycans. Glycans were enzymatically removed, as direct detection of glycoprotein can impair the sensitivity of detection by MS due to the high molecular weight and weak ionization property associated with glycans (Balaguer and Neususs, 2006; Gimenez et al., 2008; Neususs et al., 2005). Oligosaccharides on asparagine 24 were found to contain tetra-antennary structures without N-acetyllactosamine repeats and a hybrid of tetra-antennary structures with or without these repeating units while oligosaccharides on asparagine 83 of rHuEPO was found to contain tetra-antennary structures without N-acetyllactosamine repeats (Sasaki et al., 1988). This motif is absent in eEPO, although both eEPO and rHuEPO contain a similar O-linked glycan at serine 126. Subsequently, more effective ionization methods, such as MALDI and ESI were used for the analysis of the eEPO and rHuEPO. It must be noted that the ionization features of the glycoproteins are influenced by the chemistry of the carbohydrate residues. During MALDI process, there is a certain degree of dissociation of acidic glycans (Zaia, 2010). For example, the ionization of the glycosylated protein will be lesser compared to the unmodified protein that forms more positive ions. Thus, the more extensively glycosylated rHuEPO such as darbepoetin must be analyzed under the same pH values with those of less extensively glycosylated protein throughout MALDI procedure, to minimize bias in the analyses due to ion suppression. Great care must be taken when analyzing the mass spectra of MALDI as the observed ions may have dissociated, losing its residues prior to detection. To further improve the analysis, permethylation can be done to increase the MS ionization response and hydrophobicity of the glycans. Permethylation increases the stability of the glycans so that dissociation will not occur in the MALDI source. This problem of sugar residues dissociation is not observed in ESI, but this method causes complex ionization pattern.

Apart from N-linked glycans, O-linked glycans are also targeted for analysis. In one study, glycoproteins immobilized on the surface of a PVDF membrane were treated with PNGase F to release the N-oligosaccharide side chains (Jensen et al., 2012). In addition to the N-linked oligosaccharides, O-glycans were also released for analysis by reductive β-elimination. After salt removal, separation and analyses were performed with porous graphitized carbon liquid chromatography–electrospray ionization tandem mass spectrometry (PGC-LC–ESI-MS/MS), which provided information about the site variation of the glycans. The high resolving capacity of PCG has the advantage of producing good resolution. For more exhaustive information about the oligosaccharides present, different types of enzymes can be utilized for digestion (Jensen et al., 2012). Apart from glycans, another form of rHuEPOs analysis involves glycopeptides. To perform this analysis, enzymatic release of glycans is omitted and the EPO analogs are subjected only to enzymatic treatment (e.g., by trypsin) to digest the intact protein to provide peptide or glycopeptide fragments. These fragments are then analyzed by MALDI-Time-of-flight (TOF) to provide MS profiles of the fragments, which can be distinct between eEPO and rHuEPO (Zhou et al., 1998).

Unique peptide fragments (peptide markers) also can be used as effective diagnostic markers (Zhou et al., 2013). In one experiment, EPOs were first captured by anti-EPO antibody and then subjected to trypsin digestion. The extracts were purified and concentrated via an on-line trap column in the nano-LC system. The unique peptide segment, T6 (VNFYAWK) of rHuEPO, darbepoetin, and methoxy polyethylene glycol-epoetin beta (Mircera) was detected using LC-nano-ESI-MS/MS. In this analysis, which involves equine samples, rHuEPO, darbepoetin, and methoxy polyethylene glycol-epoetin beta (Mircera) were quantified at 0.1, 0.2, and 1.0 ng/mL (Yu et al., 2010).

Peginesatide, which is a member of the new generation of erythropoiesis-stimulating agents (ESAs), is a 45 kDa polyethylene glycol (PEG)-ylated homodimeric peptide that contains less sequence homology with EPO. Thus, detection of Peginesatide based on MS spectra is easier compared to other rHuEPOs that have similar amino acid sequences with the eEPO. To detect the presence of peginesatide (which is also abused by athletes), Moller et al. (2012) performed protein precipitation with acetonitrile, followed by acetonitrile removal using reduced pressure. The sample was subjected to proteolytic digestion, and the resulting products were purified and concentrated with solid-phase extraction on a strong cation-exchange resin. The product was then analyzed by LC-MS/MS with a detection limit of 0.5 ng/ml (Moller et al., 2012).

To obtain-well defined spectra, preconcentration of the target protein EPO prior to analysis can be performed via lectin-based affinity chromatography purification. Various lectins can be used for EPO purification, such as concanavalin a, jacalin and wheat germ agglutinin (WGA) (Wang et al., 2006; Yang et al., 2005). In addition, samples obtained (such as serum or urine) must be purified as the presence of salt, non-surfactant additives or contaminants can skew the analysis of the spectra. Though direct detection of peptide/glycan using MS and HPLC is an accurate method of detecting the presence of rHuEPO, these methods are expensive and involve tedious sample preparation (Methlie et al., 2013). Another method of directly detecting rHuEPO, such as by monitoring the migration rate of rHuEPO as compared to eEPO is also an elegant strategy.

6. Differential migration-based molecular discrimination between rHuEPO and EPO

The difference in the extent of glycosylation of rHuEPOs and eEPO, which is responsible for the disparate apparent molecular weights, results in differential migration of rHuEPO and EPO when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). EPO migrates at approximately 34 kDa, whereas rHuEPOs such as epoetin beta and alpha migrate at 36–38 kDa (Kung and Goldwasser, 1997; Desharnais et al., 2013). Other rHuEPOs such as Nesp and Mircera migrate at apparent molecular weights of 44–45 kDa and 69–78 kDa, respectively. To obtain a clear profile of separation, purification of EPO from urine (which contains a high amount of protein) must be executed prior to analysis by SDS-PAGE.

In an effort to augment the half-life of rHuEPOs for delayed renal clearance, PEGylation was performed. One such PEGylated protein is Mircera, which is a type of continuous EPO receptor activator (CERA). SDS-PAGE analysis of Mircera followed by Western blot detection using anti-EPO monoclonal antibody will result in a distinct band at a different molecular weight from that of other rHuEPOs. However, the detection sensitivity of this protein is lower than that of other rHuEPOs due to the interaction between SDS and PEG, which diminishes the binding affinity of the monoclonal anti-EPO antibody (clone AE7A5) against the protein. This problem was alleviated by replacing SDS with sarcosyl, which binds only to the amino acid chain of PEG and does not interfere with binding of the antibody against the target protein. As a result, higher resolution of the electrophoretic band was produced, indicating that SARCOSYL-PAGE-Western blot is an effective separation method for PEGylated rHuEPOs (Reichel, 2012a, 2012b). Leuenberger et al. (2011) reported that SARCOSYL-PAGE could be six times more sensitive than the IEF method without any

compromise of sensitivity in detecting CERA present in the athlete's blood (Leuenberger et al., 2011).

Capillary electrophoresis is another excellent electrophoresis method that provides very high resolution with small sample consumption within a short period of time (Zhao and Chen, 2014). de Kort et al. (2012) combined this method with native fluorescence (Flu) to enhance the sensitivity of detection. Flu is based on the inherent fluorescent properties of tryptophan and tyrosine of the protein, it provides information about the protein conformation without the need for fluorescent labeling of the protein. As Flu of EPO yields a higher signal-to-noise ratio compared to that of UV absorbance, the resolution achieved produced a clear glycoform pattern that enabled better discrimination between different rHuEPOs such as epoetin beta and rHuEPO-alpha (de Kort et al., 2012). To accommodate analysis of more samples (up to 120), modifications were made using double-sized gels containing 48–120 wells and three electrodes (Reichel, 2012a, 2012b). Another form of direct detection via electrophoresis is two-dimensional (2D) electrophoresis, which combines both IEF and SDS-PAGE. In this method, IEF ensures the separation of proteins by their isoelectric point (pI), while SDS-PAGE results in protein separation by molecular weight. This separation is of higher resolution compared to SDS-PAGE-based separation only (Schlags et al., 2002). IEF is combined with immunoblotting for the detection of rHuEPO, which is more accurate than IEF-SDS-PAGE analysis.

7. Isoelectric focusing (IEF)-double immunoblotting: the WADA accredited strategy

IEF-double immunoblotting is the testing method currently accepted by WADA to detect the illegal use of rHuEPO. The purpose of this combined method is to reduce non-specific binding of the secondary antibody (Lasne and de Ceaurriz, 2000). This method can detect very subtle differences in the extent of glycosylation, such as the heterogeneity in the three N-linked and one O-linked oligosaccharide side chains of eEPO and rHuEPO (Debeljak and Sytkowski, 2012). The extent of glycosylation creates a difference in charge and results in distinct pI values for each of the EPO species. Human eEPO has a pI of 3.7–4.7, whereas EPO alpha and beta have pIs of 4.4–5.1 (Wide and Bengtsson, 1990). Darbepoetin alpha has two additional glycosylation sites, which causes the pI to shift to the acidic range of 3.7–4.0.

The variation in the pI values account for the different electrophoretic mobilities of the EPOs, which can be analyzed via IEF-double immunoblotting. Lasne and de Ceaurriz (2000) used this technique to detect rHuEPO in urine. This method is not compromised by the low amount of EPO in urine. In fact, for increased sensitivity of IEF-double-immunoblotting, the requirement of up to 1000-fold concentration of the specimen is needed as the failure to concentrate the specimen leads to about 20% of the undetectable cases of EPO (Peltre and Thormann, 2003). Hence, a large volume of human urine and concentration of urine via two-step ultrafiltration must be performed prior to the IEF. Ultrafiltration involves the use of filters with a 30 kDa molecular weight cut-off value. Before the ultrafiltration step, the urine must be pre-treated by vacuum-assisted microfiltration and centrifugal sedimentation, adjusted to pH 7.4, and treated with a protease inhibitor to prevent protease-mediated EPO degradation. The amount of urinary protein added can be quantified by ELISA and should not be very high in order to attain good resolution of the IEF-double immunoblotting. Before urinary proteins are loaded onto the gel, the urinary retentates are heated at 80 °C for 3 min to inactivate the proteolytic activity.

After electrophoretic separation of the EPOs, Western blotting is performed. The proteins that are separated according to the pI

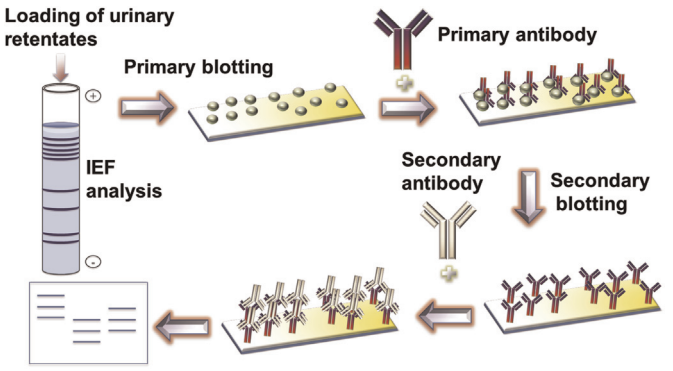

Fig. 4. Cartoon illustration of the IEF-double immunoblotting procedure. The urinary retentates are separated by IEF, which is followed by double immunoblotting. Primary immunoblotting is carried out to transfer the separated proteins to the first membrane, which then is incubated with the primary antibodies. Secondary blotting, which involves the transfer of only the primary antibodies to the second membrane, takes placed, followed by incubation with secondary antibodies. Signal detection by chemiluminescence via a CCD camera or X-ray film reveals the pattern of migration of the EPO.

are transferred to the membrane and incubated with primary antibody solution (monoclonal anti-EPO antibody). To prevent non-specific binding, which often is exhibited by the secondary antibody, the anti-EPO antibody bound to the EPO on the membrane is transferred to another PVDF membrane in a step known as secondary blotting. This process, which transfers only the anti-EPO monoclonal antibodies to the second membrane while leaving the urinary proteins on the first membrane, greatly reduces non-specific binding of the secondary antibody. Following the secondary blotting, incubation with the secondary antibody conjugated to horseradish peroxidase is conducted. The signals are detected using chemiluminescence via CCD camera or X-ray film for imaging (Fig. 4). Densitometry is used to analyze the IEF-profile.

Lasne et al. found the pattern of migration for both epoetin alpha (Erypo, Eprex) and epoetin beta (NeoRecormon) to be quite similar and within the pI range of 4.42–5.11, whereas the pI of the eEPO was more acidic at 3.92 (Lasne, 2001; Lasne and de Ceaurriz, 2000; Lasne et al., 2002). Thus, this technique enables direct detection of rHuEPO in urine, as it can be distinguished from eEPO. Lasne et al. (2009) found a different CERA to have a pI in the range of 4.8–5.2. Although it was expected to be more acidic than epoetin beta, PEG might have shielded the charges on the surface of the protein (Lasne et al., 2009). The capacity of IEF-double immunoblotting to detect the presence of any EPO variants in the urine relies on the anti-EPO antibody, which is able to form immunocomplex with most of the EPO protein domains regardless of glycosylation or modification.

8. EPO–anti-EPO immunocomplex detection

Immunocomplexing refers to the formation of a complex between an antibody and its target antigen. Protein-based probes (e.g., monoclonal and polyclonal antibodies) can recognize the corresponding targets with high specificity (Aghebati Maleki et al., 2013; de Sa et al., 2013). Hence, detection of the antigen–antibody complex (in this case EPO–anti-EPO) can be used to detect the presence of rHuEPO. Compared to polyclonal antibody, monoclonal antibody is more specific due to its single epitope recognition. Anti-EPO antibodies are used to capture target EPO from urine, as purification of EPO in its pure form and at high concentration is required prior to analysis. Spivak et al. (1977) purified EPO with more than 40% recovery from urine using wheat germ agglutinin (WGA) immobilized on agarose, but they were unable to achieve

homogeneity (Spivak et al., 1977) because lectin has high affinity for carbohydrates; thus, this lectin-mediated purification can also lead to co-purification of some other glycoproteins. Hence, purification of EPO via an agent (e.g., antibodies) that can specifically capture EPO is a more reliable technique. Utilizing polyclonal anti-EPO antibodies immobilized on the surface of a Sepharose 4B matrix, (Mi et al., 2006) were able to purify urinary EPO. Yanagawa et al. (1984) conducted EPO purification by coupling monoclonal antibody against EPO on the surface of agarose; they managed to isolate 6 mg of EPO from 700 L of urine (Yanagawa et al., 1984).

Generally, antibodies (both monoclonal and polyclonal) have been applied in numerous assays for detecting EPO, including ELISA, IEF-double-immunoblotting, and other label-free biosensor methods. For example, (Kim et al., 2006) developed an ELISA assay using anti-EPO polyclonal antibody that was able to detect 10 mU/mL of EPO. Yanagihara et al. (2008) characterized five new monoclonal antibodies against EPO and classified them into two groups: N-terminal region of EPO recognition and another group that recognizes a conformation-dependent epitope. In another study, a biosensor was developed using anti-EPO monoclonal antibody and anti-EPO polyclonal antibody conjugated on the surface of a carbon nanotube (CNT). The formation of a sandwich configuration between the anti-EPO-conjugated CNT and the anti-EPO coupled with EPO enhanced the surface plasmon resonance (SPR) signal. As a result, a dynamic range of detection of EPO (0.1–1000 ng/mL) was reported (Lee et al., 2011). Antibody is also applied in paper-based diagnostic assay, which is the frontier of point-of-care diagnostic, such as in lateral flow test. The available anti-EPO antibodies generated against EPO interact with both eEPO and rHuEPO, which fails to differentiate between these two substances. As such, the available antibody-based kits in the market (such as ELISA) can only facilitate the direct quantification of EPO in human serum/plasma, which involves both eEPO and rHuEPO. Hence, oligosaccharide-specific antibodies must be isolated for the specific detection of rHuEPO, which can expedite the development of rHuEPO-specific detection kit. Hence, the currently available antibodies are more compliant for the purpose of capturing or concentrating EPO (both eEPO and rHuEPO) prior to analysis such as electrophoresis, mass spectrophotometric analysis or lateral flow assay.

9. EPO wheat germ agglutinin (WGA) Membrane Assisted Isoform ImmunoAssay (MAIIA): a lateral flow test that rivals IEF-double immunoblotting

The lateral flow test has been the crux of many point-of-care diagnostics (Jorgensen et al., 2013; Karakus and Salih, 2013; Lawn et al., 2013). Likewise, Lonnberg et al. (2012) have devised a lateral flow test for rHuEPO detection that is more sensitive than IEF-double-immunoblotting. Known as EPO WGA MAIIA, this test consists of a strip that contains a WGA zone and an anti-EPO antibody immobilized zone (Fig. 5a). Immersion of this strip into a sample containing EPO results in EPO being captured by WGA and this EPO is desorbed via competition with N-acetylglucosamine, which subsequently displaces EPO and binds WGA. The desorption is halted by cutting-off the WGA zone, whereby the EPO bound to the anti-EPO zone (anti-EPO antibody) interacts with anti-EPO antibody bound to a carbon black nanostring. The signal, which varies from grey to black, is quantified by an image scanner and compared with the signal acquired from the standard rHuEPO. The detection of eEPO can be differentiated from that of rHuEPO based on the difference in the binding affinity of these EPOs against the lectin. The binding affinity of the eEPO against lectin is less compared to rHuEPO, as a result the signal intensity is weaker compared to the latter. The total amount of time required for the

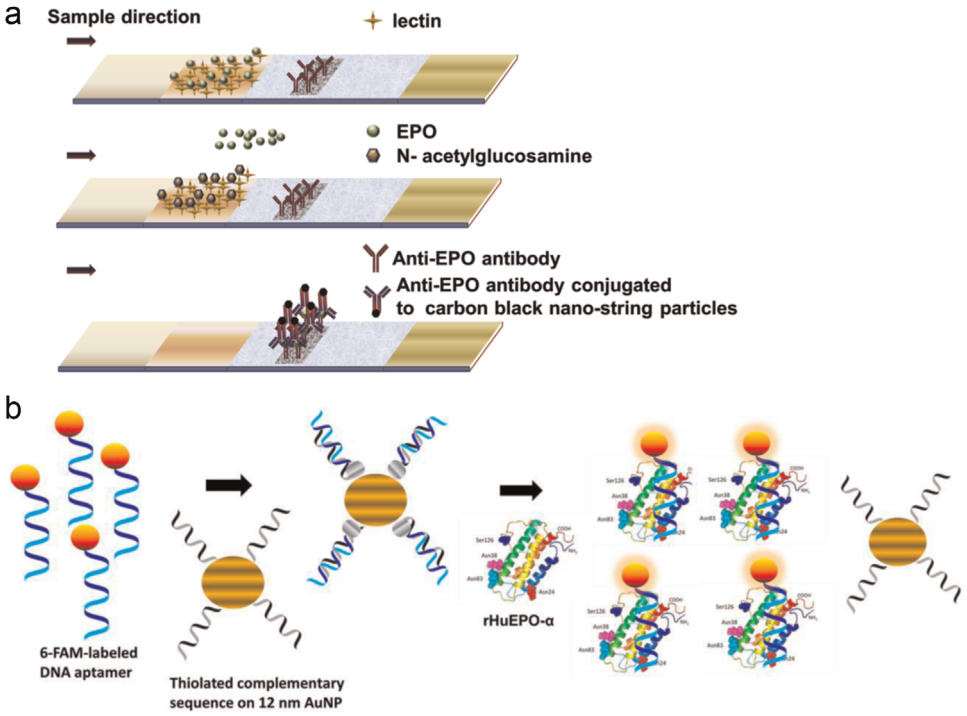

Fig. 5. (a) EPO WGA MAIIA-based detection of rHuEPO. The strip consists of two zones: the WGA zone and the anti-EPO antibody immobilized zone. The EPO captured from the urine sample by WGA is released due to competition with N-acetylglucosamine. The released EPO is captured by the anti-EPO antibody zone and then binds to the anti-EPO antibody, which is bound to a carbon black nanostring that produces a grey to black signal. The binding affinity of eEPO against WGA is less than that of rHuEPO, which enables detection of rHuEPO (if present) based on the difference in the signal intensity. (b) Gold nanoparticle (AuNP) fluorescent probe-based detection of rHuEPO. In the absence of the target protein, the 6-FAM-labeled DNA aptamer binds its thiolated complementary sequence immobilized on the AuNP surface, which acts as nanoquencher to quench fluorescent emission. In the presence of the target protein, the target interacts with the DNA aptamer, which results in fluorescence emission due to the absence of the quenching effect.

detection process is 30 min. For a more clear-cut result, the EPO can be purified first using anti-EPO antibody as the capturing agent (Lonnberg et al., 2012). Ashenden and colleagues have reported that this EPO WGA MAIIA has the capacity to detect epoetin beta even after 72 h of administration (Ashenden et al., 2012). As a dipstick-based test, EPO WGA MAIIA is very simple, fast and is applicable for both urine and blood samples (Ashenden et al., 2012). This assay has successfully discriminated different rHuEPOs (including epoetin alpha, omega, delta, darbepoetin alpha, Aranesp, and Mircera) from eEPO based on the differential affinity of the EPOs to WGA, as eEPO binds WGA with weaker affinity compared to other rHuEPOs. Another major advantage of this system is that the detection limit is as low as 0.1–1 ng/L (3–30 fmol) of EPO. The capacity to detect picogram amount of EPO in urine is crucial for EPO doping detection (Lonnberg et al., 2012). As an alternative probe, researchers also look into the potentiality of the aptamer as the nucleic acid-based probe towards detecting EPO abuse.

10. Chemical antibody-EPO complex-based detection

Aptamers or chemical antibodies are nucleic acid-based probes that are exceptionally specific. Aptamers are single-stranded DNA or RNA that recognizes a wide variety of targets with high affinity and specificity (Ellington and Szostak, 1992; Gold et al., 2012). Therefore, detecting the formation of a complex between an aptamer and rHuEPO/EPO is an alternative to immunocomplex-based detection. Compared to antibodies, aptamers have lower molecular weight, can be functionalized more easily, and exhibit no batch-to-batch variation. These artificial molecules are generated by a process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX).

Numerous aptamer-based assays have been developed for diagnostic purposes (Citartan et al., 2012). For example, Zhang et al. (2010) generated a DNA aptamer against rHuEPO-alpha that had a dissociation constant value of 39 ± 27 nM. The aptamer was generated via the SELEX process with the aid of lectin, which can selectively bind oligosaccharide side chains of EPO to expose the protein domain of EPO. Sun et al. (2011) have harnessed this aptamer to develop biosensor based on a gold nanoparticle (AuNP) fluorescent probe (Fig. 5b). In this assay, when the target protein is absent, the DNA aptamer conjugated to carboxymethylfluorescein (FAM) binds its complementary sequence on the surface of the 12 nm AuNP and fluorescence is quenched. In the presence of the rHuEPO-alpha, however, the DNA aptamer dehybridizes from the complementary aptamer sequence and binds the target, resulting in the emission of fluorescence. This assay can be performed within a few hours and has a detection limit of 0.92 nM (Sun et al., 2011).

In another study, Tang et al. (2010) developed a magnetic bead-based aptameric real-time PCR assay with a detection limit of 1 pmol/L rHuEPO-alpha (Fig. 6a). In this assay, two detection approaches (recognition-after-hybridization and recognition-before-hybridization) were used. On the other hand, Zhang et al. (2010) have demonstrated that aptameric molecular beacon (MB)-based probe can also be applied for the detection of rHuEPO-alpha. Two modes termed "Signal-off" and "Signal-on" were concocted. In the signal-off mode, the absence of the target protein promotes the binding of the DNA aptamer to the DNA sequence conjugated to the quencher (Q-DNA). This caused the quencher group to be in close proximity to the fluorophore group, quenching the fluorescence. In contrast, in the presence of rHuEPO-alpha, the target binds DNA aptamer, releasing the Q-DNA and resulting in fluorescence emission (signal-on mode). The limit of detection was 0.4 nM (Zhang et al., 2009). This DNA aptamer was also tested in

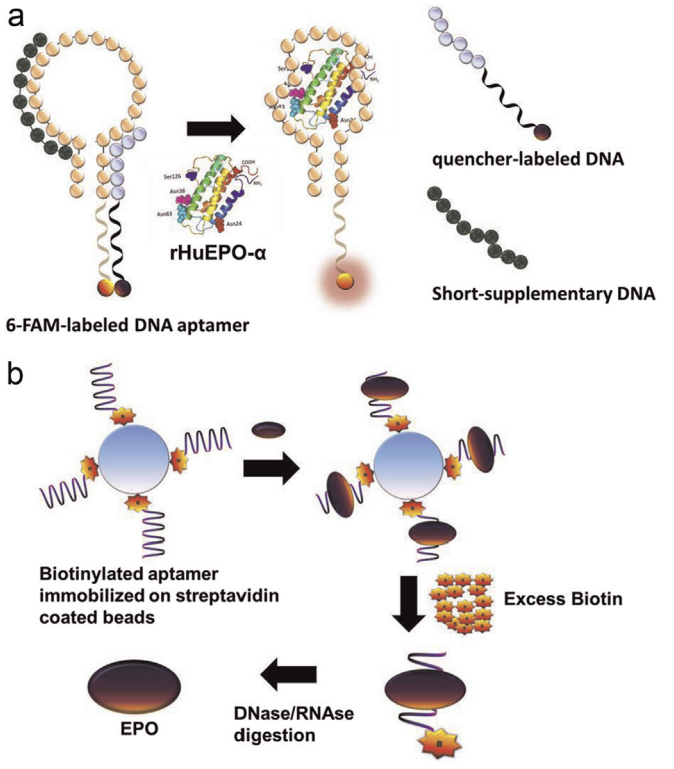

Fig. 6. (a) Magnetic bead-based aptameric real-time PCR assay. In the absence of the target protein, the DNA aptamer forms a duplex with quencher-conjugated DNA (Q-DNA). However, when the target is present, rHuEPO-alpha binds with the DNA aptamer and releases Q-DNA to permit fluorescence emission. Short supplementary DNA helps the loop region of the DNA aptamer to be exposed to the target protein rHuEPO-α. (b) Applicability of the aptamer in capturing EPO from urine. The aptamer immobilized on streptavidin-coated beads captures EPO, and elution with an excess amount of biotin elutes the EPO-aptamer complex. RNase/DNase digestion results in the release of EPO.

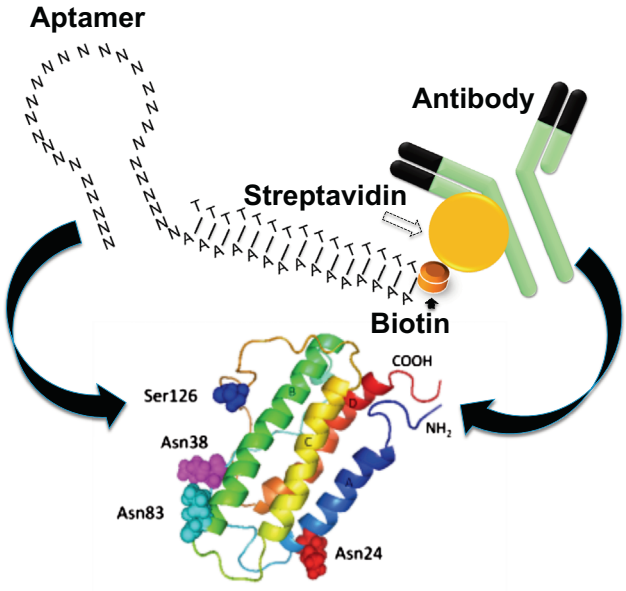

Fig. 7. Proposed strategy of aptamer-antibody chimera construct. Detection limit of EPO can be possibly enhanced if both the aptamer and antibody of the construct binds at distinct regions of the EPO protein. The aptamer functionalized with biotin is connected to the streptavidin conjugated to anti-EPO antibody. Both the aptamer and antibody that interact with different regions of EPO can capture the target protein. The length of the A and T-tail can vary, depending on the detection/capturing limit of the aptamer-antibody chimera construct.

Table 1
Summary of the assay time, specificity, sensitivity and specimen involved in the rHuEPO detection methods.

Assay	Assay time	Sensitivity	Specificity	Specimen
Indirect method				
Hematologic parameter-based measurement: i) macrocytic hypochromatic erythrocytes ii) soluble transferrin receptor (sTfR) iii) hematocrit iv) athlete blood passport (ABP)	1–2 days, varies with the technique adopted	Enables detection even after a week of rHuEPO administration Macrocytic hypochromatic erythrocytes: i) Individuals with very little rHuEPO uptake is indiscernible from iron deficiency anemia patient ii) For reliable cut-off value, analysis with more athletes of different race, sport, and gender is required sTfR: i) Highly sensitive, but can be biased due to different level of iron in the body ii) Level is higher in anemia individual, individuals living in higher altitude and with higher erythropoiesis activity ii) Assay modified to include ferritin level and red blood cell level, which can also be influenced by iron level in the body and exercise Hematocrit: i) Threshold values set for male is 50, while for female is 47% ii) Plasma volume changes, fluid loss, or conditions such as genetically determined polycythemias and iron metabolism can influence the value iii) Influenced by gender, age, body weight, and blood volume iv) Different values with manual and automated hematocrit ABP: Highly sensitive as it contains hematocrit, hemoglobin, RBC count, percentage of reticulocytes, reticulocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (multiple parameters)	Indirect detection of rHuEPO, whereby exceeding of the cut-off value indicates rHuEPO uptake For precise and reliable result, transversal and longitudinal evaluation are required	Blood
Gene expression pattern: i) mRNA ii) non-polyadenylated mRNA iii) miRNA	2–4 h, varies with the technique adopted	Based on the upregulation of the genes (mRNA, non-polyadenylated mRNA, miRNA) following rHuEPO uptake Profiling of these gene can be achieved using microarray and PCR analysis Affected by inter-individual variation that depends on ethnicity, age, gender, health status, mode of exercise, medication, nutrition and natural stimulants Inter-individual variation can be corrected by Bayesian statistics For low abundance gene identification, deep-sequencing analysis can be performed	Specific detection of rHuEPO based on the differential expression of the genes between normal individual and individual that have administered rHuEPO	Tissue/Blood/Urine
Direct method				
Glycan and peptide markers: i) High performance liquid chromatography (HPLC) ii) anion exchange chromatography iii) Mass spectrometry (MS): a) fast atom bombardment ionization (FAB) b) matrix-assisted laser desorption ionization (MALDI) c) electrospray ionization (ESI)	2 h–2 days, varies with the technique adopted and sample preparation method	Depends on the amount of sample that affects the efficiency of derivatization Detection sensitivity based on the mass and abundance profiles of the glycans In MALDI, dissociation of the glycans occurs. The invariability caused by this dissociation can be obviated by using the same pH values for all the samples to avoid skewed result. For increased MS ionization response and hydrophobicity of the glycans, permethylation can be used For higher resolution of the spectra, porous graphitized carbon liquid chromatography–electrospray ionization tandem mass spectrometry (PGC-LC-ESI-MS/MS can be utilized	Specific detection of rHuEPO: Certain EPO species contains specific sugar motifs: For example in rHuEPO, oligosaccharides on asparagine 83 was found to contain tetra-antennary structures without N-acetyllactosamine repeats, while oligosaccharides on asparagine 24 was found to contain tetra-antennary structures without N-acetyllactosamine repeats and a hybrid of tetra-antennary structures with or without these repeating units	Blood/Urine

Table 1 (*continued*)

Assay	Assay time	Sensitivity	Specificity	Specimen
		Using LC-nano-ESI-MS/MS, the detection limit of rHuEPO, darbepoetin and MIRCERA is 0.1, 0.2, and 1.0 ng/mL, respectively LC-MS/MS resulted in the detection limit of 0.5 ng/ml for Peginesatide For higher sensitivity, preconcentration of EPO prior to analysis with lectin-based affinity chromatography purification (Wheat Germ Agglutinin (WGA), concanavalin a and jacalin) is recommended The presence of salt, nonsurfactant additives or contaminants must be minimized for more sensitive detection		
Differential migration-based molecular discrimination i) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ii) SARCOSYL-PAGE-Western blot iii) Capillary electrophoresis	1 h to 2 days, varies with the technique adopted	SDS-PAGE : i) Purification of EPO from urine prior to analysis is required due to the presence of other proteins SDS-PAGE-western blotting: ii) Decreased sensitivity with PEGylated EPO due to diminished binding affinity between the protein and antibody iii) Binding affinity improves with sarcosyl which binds only to the amino acid chain of PEG (substitutes SDS) Capillary electrophoresis: i) High resolution with small sample consumption within a short period of time ii) Coupling with native fluorescence detection (Flu) enhanced detection due to higher signal-to-noise ratio	Specific detection of rHuEPO based on differential migration of eEPO compared to rHuEPO	Blood/Urine
Isoelectric focusing (IEF)-double immunoblotting	1-2 days	High resolution separation of proteins by isoelectric point (pI) For sensitive detection, concentrated sample is required, which is achieved by using large volume of urine or via two-step ultrafiltration Can detect up to 1 ng/L of EPO in urine (sample must be concentrated 1000 times prior to analysis for a clear-cut result)	Specific detection of rHuEPO based on extent of glycosylation that creates different charge and pI values for both eEPO and rHuEPO	Urine
EPO–anti-EPO immunocomplex detection: i) anti-EPO-antibody based capture assay ii) Carbon nanotube iii) ELISA	2 h to 1 day, varies with the technique adopted	Sensitivity depends on the binding affinity of the anti-EPO antibody In ELISA assay, sensitivity of detection achieved is 10 mU/mL of EPO In carbon nanotube (CNT) sensor (anti-EPO monoclonal antibody and anti-EPO polyclonal antibody) detection limit achieved is 0.1–1000 ng/mL	Detection of both eEPO and rHuEPO For specific detection of rHuEPO, oligosaccharide-specific antibodies must be isolated	Blood/Urine
EPO wheat germ agglutinin (WGA) Membrane Assisted Isoform ImmunoAssay (MAIIA):	30 min	Highly sensitive, detecting up to 0.1–1 ng/L (3–30 fmol) of EPO	Specific detection of rHuEPO based on the differential affinity of the WGA against the target protein Signal intensity of eEPO is less compared to rHuEPO	Blood/Urine
Aptamer: i) aptamer-based capture assay ii) Gold nanoparticle (AuNP) fluorescent probe-based assay iii) Magnetic bead-based aptameric real-time PCR assay iv) Aptameric molecular beacon (MB)-based probe v) Enzyme linked aptamer assay (ELAA)	2 h to 1 day, varies with the technique adopted	Depends on the binding affinity of the aptamer AuNP-fluorescent probe-based assay: i) Detection limit achieved is 0.92 nM Magnetic bead-based aptameric real-time PCR assay: i) Detection limit achieved is 1 pmol/L of rHuEPO Aptameric molecular beacon (MB)-based probe: i) Detection limit achieved is 0.4 nM	Detection of both eEPO and rHuEPO For specific detection of rHuEPO, carbohydrate-subspecific aptamer using boronic acid modified nucleic acid pool can be isolated by SELEX	Blood/Urine

aptamer-based affinity probe capillary electrophoresis with laser-induced fluorescence detection for sensitive detection of rHuEPO-alpha. The limit of detection acquired was 0.2 nM (Shen et al., 2010).

The use of an aptamer as a modular replacement for antibody in ELISA gives rise to a process known as enzyme linked aptamer assay (ELAA) (Nie et al., 2013). As aptamers have numerous advantages over antibodies, such as reusability, low cost, and ease of functionalization (Banerjee and Nilsen-Hamilton, 2013), use of ELAA may be better than ELISA for detecting EPO. Due to its high specificity, an aptamer can also be used as the capturing agent in lieu of antibody to specifically capture EPO from urine prior to analysis. Because aptamers consist of nucleic acids as opposed to EPO, which is made of amino acids, elution of the target protein captured by the aptamer can be achieved by selective degradation of the aptamer (such as RNase/DNase digestion) (Fig. 6b).

One proposed strategy is to use both aptamer and antibody against EPO in a construct known as aptamer-antibody chimera construct. Given that the binding sites of the aptamer and the antibody are on the distinct regions of the EPO protein, detection limit of EPO can be drastically enhanced by this chimeric construct. The aptamer functionalized with biotin is connected to streptavidin conjugated to anti-EPO antibody, in which both of these MREs will work in concert towards capturing the target protein EPO (Fig. 7). Just like antibody, aptamer is specific for both eEPO and rHuEPO, which capacitates aptamer to be more amenable for the EPO capturing purpose prior to analysis rather than for specific detection of rHuEPO. To generate rHuEPO-specific aptamer, nucleic acid pool conjugated with boronic acid moiety can be used in the SELEX process, as demonstrated by Li et al. (2008) that have generated aptamer against glycoprotein fibronectin.

11. Future perspectives

Detecting the abuse of rHuEPO among athletes is vital to maintain a level playing field for all the athletes and for the welfare of athletes due to its negative side effects. The main hurdle in detecting the presence of rHuEPO in a sample is its structural similarity to eEPO. Most of the available probes still fail to distinguish between rHuEPO and eEPO. This problem can be resolved by indirect method of detection. However, there are instances that the uptake of low amount of rHuEPO does not impart any changes on the level of biomarkers (hematologic parameters or gene expression level). Hence, for a more definitive result, both direct and indirect method of rHuEPO detection that can complement each other can be adopted for detection, as each of this method is associated with different assay time, specificity and sensitivity (Table 1). These strategies can also be used to detect rHuEPO that is also widely abused in enhancing the performance of the horse in racing (Bailly-Chouriberry et al., 2012). Future detection strategy should be more focused on point-of-care diagnostic (POTC), such as the development of lateral flow test so that the detection can be carried out anywhere to enable prompt decision-making.

Acknowledgments

We thank Universiti Sains Malaysia (USM) for awarding an Academic Staff Training Scheme to M. Citartan for this study, and we acknowledge the support provided by the Advanced Medical and Dental Institute, USM, to cover his traveling and subsistence costs while in Japan. T.H. Tang was supported by USM Research University Grant 1001/CIPPT/813043. Y. Chen was supported by high impact research grant from the University of Malaya: UM.C/625/1/HIR/MOHE/MED/16/5. Authors thank Research Committee of AMDI for the support of external English editing services.

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