RECOMBINANT ERYTHROPOIETIN EXTRACTION BY IMMUNOAFFINITY COLUMN AND THE DETECTION OF ISOFORMS USING CAPILLARY ELECTROPHORESIS

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By

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LIST OF ABBREVIATION

CH_3COOH	Acetic acid
NH_2	Amine group
N-terminal	Amino-terminal
AAS	Anabolic androgenic steroids
Arb	Arbitrary
Asn	Asparagine
BHK	Baby hamster kidney
BC	Before century
BCA	Bicinchoninic acid
BRP	Biological reference product
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
C-terminal	Carboxyl-terminal
CD	Circular dichroism
CV	Coefficient of variation
Con.	concentration
CERA	Continuous erythropoietin receptor activator
Cu^{+2}	Cupric
Cu^{+1}	Cuprous
Cys	Cysteine
dDQ	Delta-dot Qatar
DMT	Deoxy-methyl testosterone
DNA	Deoxyribonucleic acid
DAB	Di-amino butane
DW	Distilled water
EOF	Electro-osmotic flow

ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
FSH	Follucle-stimulating hormone
GIA	Gemological institute of America
GST	General separation transform
GFP	Green fluorescent protein
CHO	Hamster cell lines
HPCE	High performace capillary electrophoresis
HPLC	High performance liquid chromatography
HTS	High throughput screening
HCG	Human chorionic gonadotropins
hEPO	Human erythropoietin
hGH	Human growth hormone
HCL	Hydrochloric acid
IAC	Immunoaffinity column
IA	Immunoassay
IGF	Insuline growth factor
IOC	International Olympic Committee
IVD	In vitro diagnostic
LIF	Laser-induced fluorescence
LOD	Limit of detection
LOQ	Limit of quantification
IEF	Isoelectric focusing
IRMS	Isotope ratio mass spectrometery
LH	Luteinizing hormone
MS	Mass spectrometry
MGF	Mechono growth factor
mRNA	Messenger ribonucleic acid
MT	Migration time
NESP	Novel erythropoietin stimulating protein
No.	Number

PVDF	Polyvinyldifuride
PBS	Phosphate buffer solution
rEPO	Recombinant erythropoietin
rhEPO	Recombinant human erythropoietin
rhGH	Recombinant human growth hormone
RCF	Relative centrifugal force
RMT	Relative migration time
RSD	Relative standard deviation
R&D	Research and diagnostic
Ser	Serine
CH3COONa	Sodium acetate
NaN_3	Sodium azide
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaH_2PO_4	Sodium phosphate
STD	Standard
SD	Standard deviation
THG	Tetra-hydro-gestrinone
TSH	Thyroid stimulating hormone
UV	Ultraviolet
UV-vis	Ultra violate-visible
UK	United kingdom
UPD	Urine precipitate dissolvation
Fab	Variable region of the antibody
Vol.	Volume
VO_2-max	Volume-oxygen maximum (maximal oxygen consumption)
WADA	World anti-doping agency
WR	Working reagent

LIST OF SYMBOLES/UNITS

A	Absorption
A_{280}	Absorption at 280 nm
kA	Affinity constant/association constant
A	Alpha
B	Beta
^{o}C	Celsius
cm	Centimeters
R^2	Coefficient of determination
G	Grams/Gravity
>	Greater than
IU	International Unit
Ip	Isoelectric point
kDa/Da	Kilo-Dalton/Dalton
kV	Kilo-Volt
<	Less than
≤	Less than or Equal to
M	Meter
µg	Microgram
µL	Microliter
mg	Milligram
mIU	Milli-International Unit
mL	Milliliter
mM	Milli-molar
min	Minutes
M	Molar

ng/mL	Nanogram per milliliter
nm	Nano-meter
%	Percent sign
psi	Pounds (force) per square inch of area
V	Volume
Z	Zeta

PENGEKSYRAKAN REITROPOIETIN REKONBINAN MENGGUNA KOLUM IMUNOAFINITI DAN PENGESANAN ISOFORM MELALUI ELEKTROFORESIS KAPILARI

ABSTRAK

Eritropoietin (EPO) adalah suatu hormone glikoprotein endogenous. Ia disintesiskan di dalam buah pinggang dan dirembes oleh organ yang sama. Dalam pasaran, terdapat sebilangan bahan farmaseutikal termasuk EPO rekombinan (rEPO) yang mempunyai kegunaan klinikal. Dengan perkembangan teknologi novel dalam bidang farmaseutikal dan perubatan, "EPO biosimilar" telah diperkenalkan ke dalam pasaran. Sungguhpun EPO rekombinan telah luas digunakan untuk merawat pelbagai jenis anemia, ia juga telah menjadi drug yang terkenal oleh kerana penyelahgunaannya dalam sukan berprestasi tinggi. Pihak berkuasa sukan telah melarang penggunaan rEPO semenjak 1989. Penyelidikan ini telah mengguna beberapa teknik untuk penulenan EPO semulajadi serta juga bentuk EPO rekombinan dari air kencing. Dua teknik kolum imunoafiniti (IAC) telah digunakan untuk penulenan EPO; ini adalah "HiTrap Affinity Column" dan "MAIIA EPO Purification Kit". Kedua kolum berjaya digunakan untuk menahan molekul EPO. Pemulihan semula EPO dari "HiTrap column" didapati tidak berkesan manakala pemulihan semula menggunakan "MAIIA affinity column" agak memuaskan.

Penyelidikan ini juga menghuraikan suatu kaedah pencirian yang novel mengguna Elektroforesis Kapilari (CE) yang dapat digunakan dalam Kawalan Kualiti

farmaseutikal dan telah diselidik dari segi keupayaannya untk mengesan penyelahgunaan drug dalam sukan. Kaedah CE konvensional biasanya mengguna suatu label yang terikat pada EPO untuk tujuan pengesanan. Pendekatan ini sungguhpun sensitif, berupaya melibatkan bias ke dalam analisis dan ini boleh menjejas perbandingan analisis diantara rEPO dan EPO semulajadi. Penyelidikan ini berasaskan pendekatan, yang ringkasnya mengguna cahaya UV dan algoritma tanpa bias bagi mengurangkan masalah seperti ini. Oleh kerana kedua EPO semulajadi dan rekombinan terdapat secara fisiologikal di dalam air kencing pengguna, suatu pendekatan yang dapat membezakan diantara hormon semulajadi dan EPO rekombinan eksogenous amat diperlukan. Analisis ini mungkin dapat digunakan untuk kawalan antidoping. Baru-baru ini, pengasingan rEPO kepada populasi glikoform berasingan telah menjadi suatu cabaran besar terutamanya apabila kepekatan hormone yang agak rendah bagi ujian anti-doping. Tujuan penyelidikan ini adalah untuk membangunkan pengesanan glikoform EPO yang berkesan, sensitif dan pantas di dalam matriks air kencing. Pembangunan elektroforesis kapilari (CE) telah memainkan peranan penting dalam memperkenalkan aplikasi terkini dalam pengesanan glikoform rEPO. Walau bagaimana pun, sensitiviti kaedah ini mempunyai paras pengesanan LOD pada 3000 IU, dan ini menghadkan penggunaannya dalam analisis antidoping oleh kerana paras EPO yang begitu rendah dalam air kencing. Penyelidikan ini telah menunjukkan keupayaan CE dalam pengasingan kandungan glikoform yang kompleks dalam EPO dan ini mewujudkan buat kali pertamanya pendekatan CE tanpa penggunaan label bagi Pengawalan Kualiti (QA).

RECOMBINANT ERYTHROPOIETIN EXTRACTION BY IMMUNOAFFINITY COLUMN AND THE DETECTION OF ISOFORMS USING CAPILLARY ELECTROPHORESIS

ABSTRACT

Erythropoietin (EPO) is an endogenous glycoprotein hormone. It is synthesized in and secreted mostly by the kidneys. In the market, there are a number of pharmaceuticals of recombinant EPO (rEPO) for clinical use. With the advent of novel technologies in the pharmaceutical and therapeutic areas, recombinant EPO have been introduced into the market. Although recombinant EPO has been broadly used to treat various forms of anaemia, it has also become a very well known drug due to its misuse in endurance sports. Sport authorities have prohibited the use of rEPO since 1989. This work has used a number of techniques to purify natural EPO and the recombinant form from the urine. Two Immunoaffinity column techniques (IAC) were used to purify EPO i.e. HiTrap Affinity Columns and MAIIA EPO Purification Kit. Both columns were successfully able to trap EPO molecules. EPO recovery from the HiTrap columns was negligible < 1%, whereas the recovery of MAIIA affinity column was satisfactory.

The work has also developed a novel characterization tool using Capillary Electrophoresis (CE) that can be used in pharmaceutical Quality Control (QC) and has been investigated for its ability to detect drug misuse in sport. Conventional CE tools generally use a label that attaches to the EPO for identification. These approaches though sensitive, can introduce biases into an analysis that could prove inimical to a

comparison between rEPO and EPO samples. We have used a tool that simply uses UV absorption and unbiased algorithms to minimize any such problem. Since both natural and recombinant EPOs are physiologically present in urine, there is an absolute need for an approach, which can differentiate between the natural hormone and the recombinant exogenous EPO. The analysis may have some utility for anti-doping control. The separation of intact rEPO into distinct glycoform populations has recently been a great challenge to achieve particularly in low concentrations for anti-doping world. The aim of this work is to develop a sensitive, rapid and cost effective detection of rEPO glycoforms in urine matrix. The development of capillary electrophoresis (CE) has played a key role in bringing about the modern applications of rEPO glycoforms detection. However, the sensitivity of this method used has an LOD level of 3000 IU, which limits its use in sport world due to the low level of EPO in urine that is much lower than the real value. We show in this study the ability of CE in separating complex glycoform content of rEPO allowing for the first time unbiased approach to Quality Assurance (QA) using label-free approach.

CHAPTER 1

1.1 Introduction

Competition is a natural instinct in the human nature, which has been essential from both evolutionary and survival point of view (Giuseppe, et al, 2006). In sport the use of illegal drugs to deceitfully increase aerobic performance, has plagued the world of competition for ages (Grégory, et al, 2006. Osquel, et al, 2008). Doping is now a global problem that follows international sporting events worldwide (David, et al, 2007). Although athletic performance enhancement has been achieved since ancient competitions by using various diets, training routines and hard work, it can be also achieved by using a wide variety of physiological, mechanical and pharmacological doping techniques (David A et al, 2007).

In sports, doping refers to the use of performance-enhancing drugs, particularly those forbidden by organizations that regulate competitions. This definition is established by the medical commission of the International Olympic Committee (IOC) that is based on the prohibition of certain types of pharmaceuticals. It took several decades for sport organizations to realize the magnitude of the threat that doping poses to fair play, and the hazards it presents to the health and well-being of athletes. This phenomenon thereby triggered the recent establishment of the systemic fight against doping (Osquel, et al, 2008).

In 1967 the International Olympic Committee established the medical commission that launched the introduction of anti-doping regulations, including the first official list of prohibited substances (Osquel, et al, 2008). The committee has attempted to stop the spread of doping problems for the past half century with minor effects (David, et al, 2007). In 1999 the World Anti-Doping Agency (WADA) was created as a result of the IOC-convened World Conference on Doping in Sport, where both the IOC and government agreed to create an independent agency to sponsor, coordinate, and monitor the fight against doping in sports internationally. Some of the main WADA's responsibilities are the creation and implementation of the World Anti-Doping Code and the related International Standards. The code represents the foundation stone for bringing all sport anti-doping regulations of all countries together. The World Anti-Doping Code, therefore, defines doping as the incidence of an anti-doping rule abuse (Osquel, et al, 2008).

According to the code, there are three criterions in which two have to be met for a substance to be added to the Prohibited List. First, it's potentiality to enhance sport performance. Second, its health risk for the athlete. Third, sport's spirit opposition.

The performance-enhancing effects of any particular substance are: 1- Ergogenic effect that includes enhanced strength, higher energy production and better recovery. 2- Anabolic potential that includes increased protein synthesis particularly in the muscles. 3- Motivating properties by increasing attention and loss of fear. These

substances grant a competitive advantage to athletes. The Code establishes the principle of "strict liability", in which the presence of any prohibited substance or its metabolite in an athlete's specimen is adequate to represent an anti-doping rule desecration, irrespective of the athlete's personal intention for using such illegal enhancers (Osquel, et al, 2008).

There are two different origins for prohibited substances: 1- Exogenous, which are substances that cannot be naturally produced by the body. For example, synthetic Anabolic Androgenic Steroids (AAS), including methyltestosterone and nandrolone. 2- Endogenous, those are substances produced naturally by the body, such as, human growth hormone (hGH), erythropoietin (EPO), testosterone, dehydroepiandrosterone, and insulin (Osquel, et al, 2008).

In Olympic and professional sports, performance enhancement drugs have become a medical, ethical and legal problem for current athletes and athletic organizations. Although it was thought that with educational programs, testing and supportive medical treatment, drug-abusing behavior would diminish, this has not been the case. This is predominantly due to the amount of money coupled with winning the competition in today's sports industry. Because athletes are being rewarded for winning at nearly every level of competition, coaches would sacrifice and risk a great deal in order to obtain a competitive rim and enhance performance at all coasts (Osquel, et al, 2008).

1.2 Objectives

1- To establish a sensitive, selective and cost effective method for sample clean-up for EPO extraction.

2- To investigate the utility of capillary electrophoresis to isolate recombinant EPO isoforms using label-free approach on CZE, striving to provide an alternative technique to the current WADA-accredited approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Hormone Abuse in Sports

Hormone abuse by athletes may lead to serious health problems for individuals, as well as the destruction of the competition's spirit among athletes, officials and spectators in sport (M Verroken, 2001). Therefore, the abuse of hormonal substances in sport is prohibited. The higher authorities in sport attempt to establish rules and regulations in order to stop hormone abuse among athletes. They are also working on those athletes who have worked around these rules, challenging themselves to the extent that the anti-doping system itself is questioned. Testing for hormone abuse is very challenging as these hormones are also endogenously produced, for that reason, hormone analytical results requires careful review. Since some findings might be indications of some medical conditions, results interpretation needs a hypersensitive handling to obtain accurate results (M Verroken, 2001).

Athletes at the ancient Olympic Games had special diets to advance their physical capabilities. At the 668 BC Olympic Games, the winner of the 200 m sprint was said to use special diet of dried figs. This picture is not so far from the supplements that are consumed by the athletes in today's athlete's sports nutrition support programs (Finlay & Plecket 1976).

Examining the popularity of hormone abuse by theoretical perception on ethics, allows a philosophical study of the dilemmas facing sports as well as a better identification of issues that can help in resolving the problem of doping. Approaching the popularity of hormone abuse from ethical prospective puts the first step to examine what is acceptable, permitted and where the dividing lines are supposed to be drawn. For instance, in general sports medicine and medicine are governed by an ethic that is dedicated to restore and repair the form and function of human being as humanly as possible (M Verroken, 2001). The data collected from the testing programs provide one guide to the actual popularity of hormone abuse. However, since not all findings are considered doping offences, this cannot be used as a definite guide to the extent of hormone abuse. Also, as mentioned above, the use of hormone treatment with or without therapeutical indication promotes more complications to the disciplinary process. Accordingly, the door is open for the anti-doping system to be subjugated by dishonest scientists, raising challenges that examine the limit of credibility. Thus, a close collaboration between scientists and sport is needed to prevent the athletes from becoming victims of the rules proposed to protect sport (M Verroken, 2001).

2.2 Hormones as Doping Agents – Overview

2.2.1 Anabolic Androgenic Steroids

Anabolic androgenic steroids are examples of doping agent. They are related to the male hormone testosterone (Handelsman, 2006). Due to its augmenting effect on

muscle mass and strength, AAS are amongst the most frequently detected drugs in sport (Osquel, et al, 2008). Despite the serious adverse effects associated with the use of these drugs, AAS misuse by athletes and non-athletes are the same, including schoolchildren. The adverse effects include, reduced fertility and gynecomastia in males, masculinization in women, as well as cardiovascular complications, cancer, liver toxicity and behavioral disorders (Sánchez, et al, 2008). Moreover, the irreversible damage to organs occurs following chronic high-dose usage. There is also a tendency for taking harmful high doses of AAS in combination with other performance-enhancing drugs, such as human growth hormone (hGH) (Parkinson, et al, 2006).

What makes AAS an easy choice for athletes, risking their health to get an undeserved competitive advantage, are: 1- The strong and long-lasting benefits to performance obtained by these agents, 2- The documented attempts to develop new generation of sophisticated up-market AAS that are more and more difficult to detect (Osquel, et al, 2008).

There are a number of approaches that have been developed in order to detect these compounds including designer steroids, such as Tetra-Hydro-Gestrinone (THG) and Desoxy-Methyl Testosterone (DMT). The analytical methods that have been employed are based on the combination of chromatographic Liquid Chromatography or Gas Chromatography and Mass Spectrometric, as well as Isotope Ratio MS (IRMS). The WADA accredited laboratories worldwide successfully implement these methods (Osquel, et al, 2008).

The increased awareness of the anti-doping authorities that is led by WADA, in addition to the back up and growing support from the law-enforcement agencies and the pharmaceutical industry. All the forementioned in combination with the development of more sophisticated detection methods, has resulted in the successful identification and banning of newly developed performance-enhancing drugs, including designer steroids. Yet, the outcomes of these investigations have shown continued widespread use of prohibited substances by athletes, despite their awareness of the risk, which can be the termination of their sporting career as a result of anti-doping violation (Osquel, et al, 2008).

2.2.2 Peptide Hormones

In recent times, the misuse of endogenous hormones have noticeably increased as a result of a number of factors including, the development of molecular biology techniques particularly the recombinant DNA technology. This technology has pumped the market with quite cheap synthetic hormones. Accordingly, the development of this technique has contributed in the elimination of some of the risks associated with the use of purified hormones, for instance, the elimination of Greutzfeldt-Jacob disease following the administration of hGH isolated from cadaveric pituitary glands (Buchanan, et al, 1991).

The prohibited substances and their releasing factors are: 1-Erythropoietin (EPO), 2-Insulin-like Growth Factors (IGF, e.g. IGF-I and Mechono Growth Factors (MGF), 3-Gonadotrophins (e.g. Luteinising Hormonr (LH) and Human Chorionic Gonadotropins (HCG), 4-Insulin and 5-Corticotrophins. Endogenous peptide hormones with capability of performance enhancing properties are listed in WADA's prohibited substances list under S2 section "Hormones and related substances". All other substances with comparable chemical composition or comparable biological outcomes are found under this section as well (Osquel, et al, 2008).

The time window for the detection of peptide hormones is extremely short, due to their rapid degradation and clearance from the body. Additionally, the significant similarity between the endogenous (naturally produced) and exogenous (manufactured) peptide hormones, in their structure and biochemical properties, makes their detection extremely demanding and challenging. To date, the detection of these large and complex proteins is based on immunological identification of each substance by specific antibodies. Although, there have been attempts to detect these hormones by Mass Spectrometry based techniques (M Verroken, 2001, Handelsman DJ. 2006, Liu C et al. 1996).

2.2.3 Gonadotropins

Human chorionic gonadotropin (hCG) is a glycoprotein that belongs to a family of peptide hormones that also includes thyroid stimulating hormone (TSH),

follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Stenman, et al. 2004, Pierce, et al. 1981). It is produced in abundance by the placental trophoblastic cells during pregnancy that also stimulates the production of steroid hormone in the ovaries (Stenman, et al. 2004). The hormone is highly glycosylated with β-carboxy terminus. The β-carboxy terminus facilitates its detection by certain antibodies or via mass spectrometry (Gam LH, et al. 2003, Birken, et al. 2003, Birken, et al. 1982).

Human cherionic gonadotropin has been used for doping due to its stimulating effect on the production of endogenous steroid hormone that increase testosterone levels without affecting the testosterone/epitestosterone ratio. The testosterone/epitestosterone ratio is used as a criterion for detecting doping with exogenous testosterone. The use of hCG cannot be ignored, despite hCG not being widely abused (de Boer D et al. 1991, Johnson, et al, 1993).

Luteinizing Hormone is synthesized and secreted via gonadotropes of the anterior lobe of the pituitary gland. The hormone mediates its biological activity all through the same receptor as hCG (Stenman, et al. 2004). On one hand the hormone mid-cycle rush triggers ovulation and encourage the synthesis of progesterone and estrogen in females. On the other hand, it stimulates the production of testosterone by leydig cells in men (Robinson, et al. 2007, Wunsch, et al. 2007). Human menopausal gonadotrophin is a collection of FSH and LH that has the capability to be misused for performance-enhancing intentions.

At present, the existence of hCG and LH is considered a doping violation when detected above certain values in male athletes. However, in female athletes, establishing the origin of these peptide hormones whether they are exogenous or naturally produced is considered a challenging task (Osquel, et al, 2008). Human chorionic gonadotropin in particular may stay high for a number of weeks following premature spontaneous miscarriage. Therefore urinary hCG detection in female athlete may reveal unrecognized pregnancy, accordingly risking an invasion of privacy. Moreover, in disparity to men, hCG is reported to have insignificant effects on blood testosterone levels in women (Handelsam, 2006).

Urine doping analysis of hCG and LH is done by immunoassays using panels of hormone specific antibodies. Nevertheless, some of these contemporary techniques have inherent limitations. Therefore, new and more specific methods based on tandem mass spectrometry (MS/MS) and high performance liquid chromatography (HPLC) combined with MS/MS has been developed all through WADA-sponsored research (Bowers, 1997, Leinonen, 1999).

2.2.4 Human Growth Hormone

Human growth hormone (hGH) is a single chain polypeptide hormone synthesized and secreted predominantly in the somatotroph cells of the pituitary gland. The hormone is species specific that stimulates many metabolic processes in cells. It affects protein, fat, carbohydrates and mineral metabolism (Chawla, et al, 1983,

Daughaday, et al, 1965, Ehrnborg, et al, 2000). Currently, hGH is considered one of the most widely abused performance-enhancing agent (Osquel, et al, 2008).

Human growth hormone is a 22-kDa molecule that exists as a complex combination of several isoforms. The isoforms resulted from alternative mRNA splicing (20 kDa and 17.5 kDa) or as a result of proteolytic cleavage of the mature protein (DeNoto, et al, 1981, Lecomte, et al, 1987, Lewis, et al, 1978).

Recombinant hGH (rhGH) structurally and biochemically impossible to differentiate from the 22 kDa endogenous isoform. It has been found that when given to GH-deficient individuals, it increased exercise time and maximal oxygen consumption (VO_2 max) after 6 months of treatment (Beshyah, et al, 1995, Cuneo, et al, 1991). Also it increases body muscle mass, decreases body fat, increases cardiac output and improves wound healing (Ehrnborg, et al, 2000, Salomon, et al, 1989).

Detection of doping with hGH constitutes a great challenge for anti-doping laboratories. A novel approach to detect doping with rhGH, described as the isoform differential immunoassay, has been proposed by Prof. Christian Stransburger and Drs Zida Wu and Martin Bidlingmmaier (Bidlingmmaier, et al, 2000, Wu Z et al, 1999). The assay is developed to detect hGH doping by utilizing the differences in the magnitude of hGH isoforms under physiological conditions and following doping practice. It is based on the concept that the composition of hGH in blood is a

combination of different isoforms present at constant relative proportions. Whereas, the rhGH is comprised only from the 22-kDa molecular form (Osquel, et al, 2008).

Exogenous rhGH increases the concentration of the 22-kDa isoform. It also suppresses the non 22-kDa concentrations for up to 4 days. Accordingly, the natural ratios established between these hGH isoforms (Wallace, et al, 2001). Recognizing the monomeric 22-kDa hGH or a mixture of pituitary derived hGH isoforms through two different immunoassays that employ antibodies, the elevated ratios of 22-kDa hGH to pituitary derived hGH isoforms are used to designate doping with rhGH (Osquel, et al, 2008).

2.3 Erythropoietin

Erythropoietin (EPO) is an endogenous glycoprotein hormone (Jordi, et al, 2007). It was discovered in the 19th century upon observations of pallor renal diseased patients (Joanne, 2006). The observations are based on the hypothesis made by Cornot and De Flandre in 1906. They suggested that there is a hormonal factor involved in the regulation of haemopoiesis (formation of blood cells) (Jordi, et al, 2007). They used the term 'haemopoietin' to describe the hormonal substance in their study on animals. This work cemented an upcoming breakthrough made by Bondsdorff and Jalvisto 40 years later, who were able to link solely EPO with red blood cell production (Bondsdorff, et al, 1948). In the 1950s scientific and clinical findings established by Jacobson and his

co-workers were the determined factors behind the evidence, that the kidneys are the main sites of EPO production (Jacobson, et al, 1957).

In adult humans, EPO is synthesized in and secreted mostly by the kidneys (Jordi, et al, 2007). The peritubular fibroblasts of the kidney's renal cortex cells is the primary site for its synthesis, which accounts for approximately 90% of all systemic EPO (Chikuma, et al, 2000). The liver produces up to 10% of the total hormone formed in the body (Emmanuelle, et al, 2003). Also there is evidence that the brain and uterus contribute minor amounts of EPO (Chikuma, et al, 2000, Maria, et al; 2008). The hormone induces red blood cells proliferation, which increases their mass number and hence improves oxygen transport and delivery to the tissues. This improvement in oxygen delivery is achieved by increasing haemoglobin concentration within the cell, which maximizes oxygen uptake (Figure 2.1) (Emmanuelle, et al, 2003, Jordi, et al, 2007, Rafael, et al, 2003).

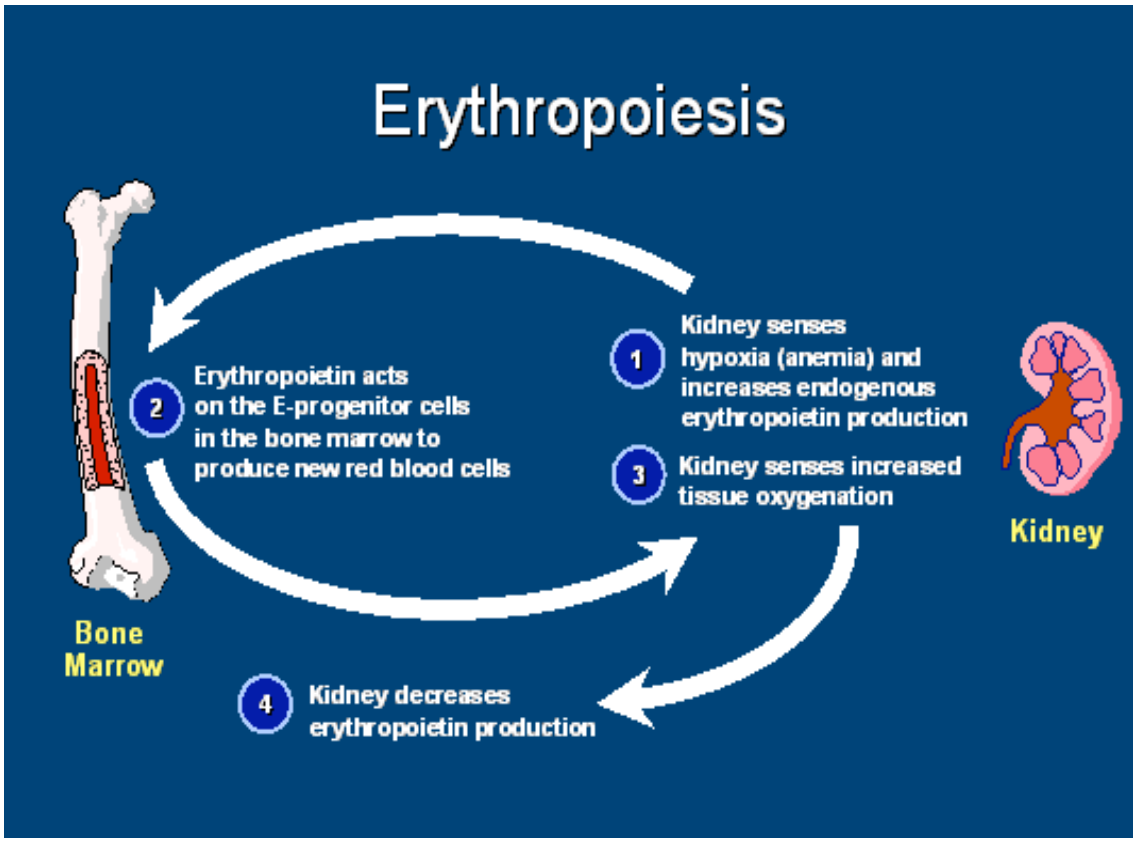

Figure 2.1: Erythrocyte production (Erythropoiesis). Adapted from http://www.hdcn.com/symp/05anna/02/pet1/pet1.htm

Moreover, EPO plays a major role in the maturation of erythroid precursor cells in the bone marrow (Osquel, et al, 2008, Venke, et al, 2001). From there, EPO is considered the main regulator of erythropoiesis (regulation of red blood cell production) (Erslev, 1991, Jordi, et al, 2007, Rafael, et al, 2003). It has a crucial physiological feedback mechanism in maintaining red blood cells number in the blood stream and in maintaining sufficient levels of oxygen supply to the tissues (Estela, et al, 2009).

2.3.1 Erythropoietin Structure

2.3.1.1 Human Erythropoietin (Endogenous)

Human EPO (hEPO) is a 30-kDa glycoprotein with a hormonal activity of 70,000 IU/mg (Maria, et al, 2008, Rafael, et al, 2003). The mature protein consists of 166-amino acid polypeptide chain with a molecular weight of of 18,398 Da for the protein moiety (Jorge, et al, 2005, Por-Hsiung, et al, 1985, Venke, el at, 2001). Nevertheless, due to the post-translation modifications, the C-terminal arginine is removed (M.A. Recny et al, 1987). The polypeptide chain contains 3 more basic amino acids than acidic ones. The molecule holds irregular distributed charged residues that have estimated to be 27% of the total protein. These residues are not found in region 77-88, whereas the NH_2- and C-terminal ends are highly charged (Por-Hsiung, et al, 1985). A fascinating feature of EPO is that, although glycine and proline residues are known to be strong breakers of α- helix and β-sheet structures, they are randomly distributed through most of the molecule. These residues are not found in regions 4-27 and 130-150. Therefore, there is a high degree of α- helix structures in these regions (Por-Hsiung, et al, 1985). The α- helix content was revealed by the near and far- UV CD spectra to be around 50%. The near and far- UV CD spectra are used to uncover the secondary structure of a molecule (Por-Hsiung, et al, 1985). Another study of the α-helix and β-sheet structures using a computer program based on method used by Chou and Fasman (Por-Hsiung L et al, 1985), α- helix content is about 36% whereas β-sheet content is about 28%. A different analysis by the method used by Garnier et al (Garnier

et al, 1978), predicts that the α- helix content is to be 42% together with β-sheet content of about 21% (Figure 2.2).

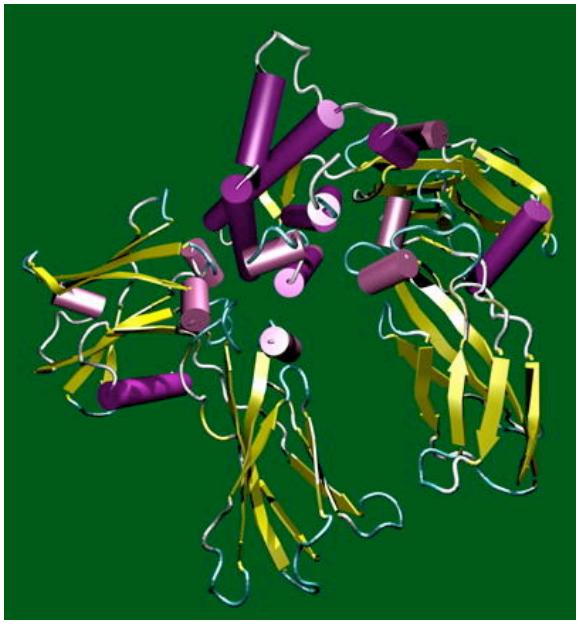

Figure 2.2: Protein structural unit of a single polypeptide chain of amino acids. The purple cylinders represent alpha helixes and the yellow arrows represent beta sheets. Adapted from http://www.sciencephoto.com/media/7778/enlarge

The glycoprotein is highly glycosylated (S.B Krantz, 1991). It has three N-glycosylation sites at Asn24, Asn38 and Asn83 and one O-linked glycosylation site at Ser126 attached to the polypeptide backbone (Figure 2.3) (J.W. Fisher, 2003, Venke, el at, 2001). The polypeptide chain synthesis of a glycoprotein is genetically regulated, whereas, the oligosaccharide chains are processed and attached by a series of enzyme reactions. Therefore, glycoproteins appear as a mixture of glycoforms, which have shown to be cell, species, polypeptide and site specific (Parekh, et al, 1987, Parekh, et al

1989, Rudd, et al, 1997, Zerfaoui, et al, 1996). Consequently, each glycoprotein has a reproducible and characteristic glycosylation pattern (Kanazawa, et al, 1999, Storring, et al, 1998). It was estimated by Wide and Bengtsson that the serum EPO contains 20-30 different forms found in the blood of anaemic patients (Lasne, 2006).

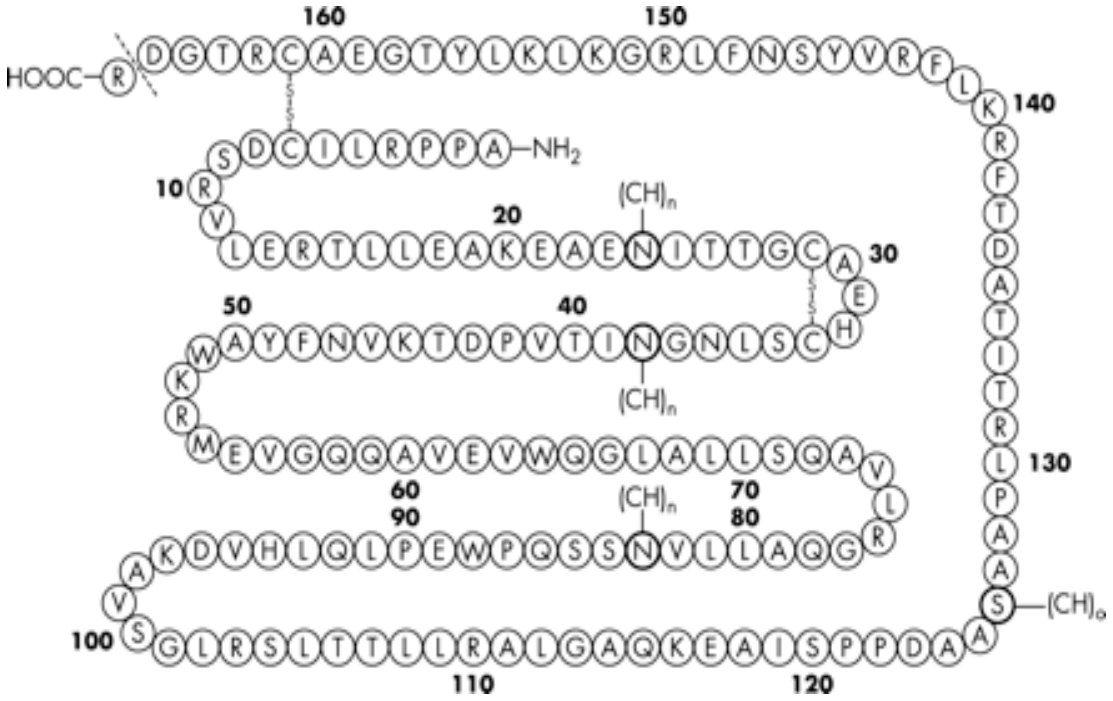

Figure 2.3: The primary structure of erythropoietin presenting the outline of 165 amino acids. There are two disulfide bonds joining the molecule together between cysteines 29 and 33, cysteines 6 and 161. Three N-lined sugars are existing at asparagines 24, 38,and 83 and one O-linked sugars is existing at serine 126. Adapted from http://www.sciencephoto.com/media/7778/enlarge

The carbohydrate content contributes to approximately 40% of the total molecular mass of the molecule (Choi, et al, 1996, Venke, et at, 2001). Glycosylation is most important for the in vivo and in vitro biologic activity of the hormone. These oligosaccharide units of the glycoproteins provide a range of functions, including

folding of generating polypeptide chains. This process takes place in the endoplasmic reticulum (Venke, et at, 2001). The endoplasmic reticulum protects the protein moieties from modulation of the biologic activities as well as from the action of proteases (Imai N et al, 1990, Rademacher, et al, 1988). Modifications or elimination of the glycan chains lead to alterations in its activities (Choi, et al, 1996, Venke, et at, 2001). Moreover, the macromolecule has a biologic marker that prevents the hormone from depuration in the liver before it reaches its physiologic target known as sialic acids. These residues are located in strategic positions of the polysaccharide chain (Choi D et al, 1996). Studies showed that the number of sialic acid residues and the N-linked oligosccharides branching pattern modify the pharmacodynamics, biologic activity and catabolism speed of the EPO molecule (Fukuda, et al, 1989, Higuchi, et al, 1992, Imai N et al, 1990). Human EPO encloses two disulfide bonds, one formed between Cys7 and Cys161, the other between Cys29 and Cys33, noting that the second disulfide bond is sandwiched between two proximate glycosylated sites, Asn24 and Asn38 (Por-Hsiung L et al, 1985). After entering the blood stream, EPO has estimated half-life of 6 to 8 hours (Adamson, 1996). The production of an in vivo active hEPO needs the biosynthetic machinery of higher cells. For that reason at present, all EPO that is on hand in the market is produced in mammalian cell culture (F.K. Lin et al, 1985, K. Jacobs et al, 1985).

2.3.1.2 Recombinant EPO (Exogenous)

There are a number of pharmaceuticals of recombinant EPO (rEPO) that are available in the market for clinical use. They are classified, according to the manufacturing methods, into epoetin alpha, epoetin beta, Novel Erythropoiesis Stimulating Protein (NESP or darbepoetin alpha), Continuous Erythropoietin Receptor Activator (CERA), and others (Venke, el at, 2001, Estela, et al, 2009). The peptide sequence of the rEPOs is homogenous to the natural or endogenous form, however they differ in their carbohydrate site (Choi, et al, 1996). This observation in the composition and disposition of the polysaccharide structure is due to the different sources of synthesis, as well as manufacturing sites from different parts of the world (River, et al, 2003).

Epoetin alpha and beta are produced by Hamster cell lines, such as the Chinese hamster ovary (CHO) and baby hamster kidney (BHK). Those two organs are the common hosts for the recombinant human glycoprotein production intended for therapeutic use. The glycosylation pattern of the proteins produced by these cells is qualitatively similar to those in human cells; however, some carbohydrate motifs are explicit for human tissues and are not synthesized by these cells. The reason for that is that they are deficient in the appropriate sugar-transferring enzymes, including sialyl-α 2-6 transferase, bisecting N-acetyl glucosamine transferase, and α 1-3/4 fucosyl transferase (Venke, et al, 2001). In human serum, the lack of tetra-acidic oligosaccharide structures is the most important difference between circulating hEPO

and rEPO as revealed by charge analysis (Venke, et al, 2001). The charge analysis shows that rEPO has a reasonably high content of tetra-sialylated oligosaccharide structures compared to circulating hEPO. Epoetin alpha contains 19% of tetra-sialylated oligosaccharide while in epotin beta the tetra-acidic glycans dominate the structure and has been estimated to be 46%. The half life of glycoproteins increases by the terminal sialic acids content. This is fundamental with respect to in vivo bioactivity of hEPO (Ashwell et al, 1974, Ashwell, et al, 1982, Fukuda, et al, 1989, Goldwassaer et al, 1974, Lowy PH et al, 1960).

2.4 Techniques to Detect Recombinant Erythropoietin

The importance and challenge of detecting EPO misuse in sports has prompted the request to develop several strategies for EPO detection (Jordi, et al, 2007). One of these strategies, developed by Parisotto et al, so-called indirect method that monitors the physiological and biochemical effects of EPO administration. This method measures blood parameters such as haematocrit, haemoglobin, and reticulocytes in the whole blood that has been extracted from athletes. This approach depends on using more than one parameter that would increase the test specificity and decrease the possibility of false positive (Jordi, et al, 2007). As a substitution, the structural differences between endogenous human EPO and recombinant EPO, which lies in the glycosylation pattern of the protein, can be used for a more definitive direct method (Jordi, et al, 2007). The heterogeneity can then be demonstrated in the form of multiple glycoforms that differ in the charge, weight and sugar composition.

2.4.1 Isoelectric Focusing

Isoelectric focusing (IEF) is the official method approved by the IOC and the WADA for rEPO detection in urine EPO test (Estela, et al, 2009). Currently, IEF is the only detection method that is capable of differentiation between endogenous and recombinant forms of EPO at low concentration levels found in biological fluids. The concentration of hEPO found in urine and/or plasma is estimated to be $\leq$ 200 ng/L (Estela, et al, 2009).

In the year 2000, Lasne and de Ceaurriz reported the first data on the method that was able to detect and differentiate between endogenous and recombinant EPO in urine. Briefly, the method consists of 4 major steps, 1- urine concentration, 2- Isoelectric Focusing (IEF) separation, 3- double blotting and 4- chemiluminescence detection (Jordi, et al, 2007). In the first step, an aliquot of 20 ml urine is concentrated by ultra-filtration to a volume of approximately 20 μl via a 30-kDa molecular mass cut-off. Then the amount collected is applied onto an IEF gel with a pH gradient from 2-6. Following that the separation of different isoforms is initiated by the applied electrical field. The separation goes on the basis of their respective isoelectric points (*Ip*). Once the separation is accomplished, the gel is subjected to a double-blotting process where all proteins are transferred from the gel to a polyvinyldifluride (PVDF) membrane. Mouse anti-EPO monoclonal antibodies are used to identify EPO, next the antibodies are detached and transferred to a second PVDF membrane. Finally, the antibodies are

identified by anti-mouse biotinylated antibody (Jordi, et al, 2007). To complete the steps, a streptavidin peroxidase complex is added to the gel in the presence of luminol and hydrogen peroxidase to generate chemiluminescence (Figure 2.4).

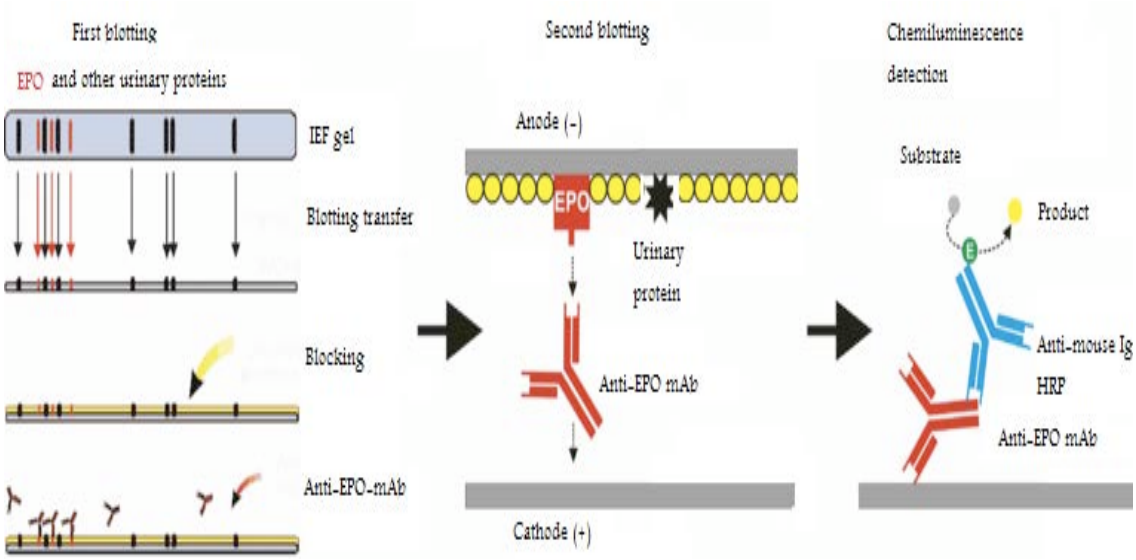

Figure 2.4: Simple diagram summaries the 1st blotting, 2nd blotting and the chemiluminescene step that visualizes the IEF bands. Adapted from (Joaquim M. 2011)

The end result of this method is a representation of a set of bands that shows groups of EPO isoforms as shown in (Figure 2.5).

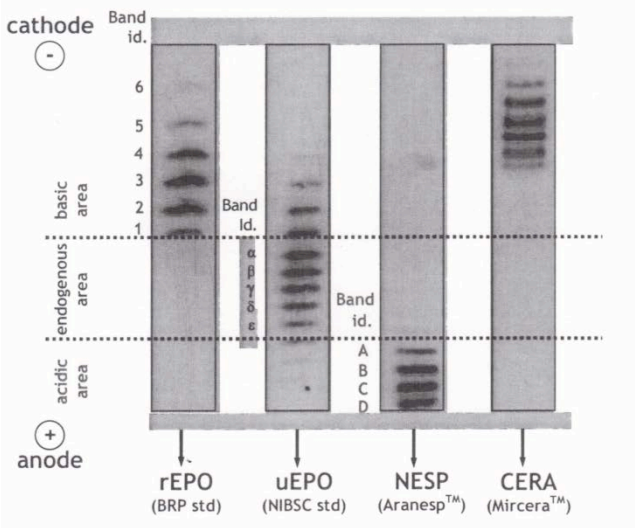

Figure 2.5: Isoelectric focusing images obtained for the analysis of recombinant EPO. Adapted from WADA technical document – TD2009EPO

On behalf WADA, Peltre and Thomman reviewed thoroughly the newly developed method and validated it despite its complexity and cost. Also Peltre and Thomman suggested some improvements in some of the steps, fields of future development and research (Jordi, et al, 2007).

During the Sydney Olympic Games in the year 2000 the IEF method for EPO was used in connection with the indirect blood method, though no sample was found to contain rEPO during that event. The weakness of this method is that the detection of rEPO bands do not have a unique pattern, nevertheless they extend through the endogenously EPO *pI* values. Accordingly, there was a need to establish a certain objective criteria for the presence and identification of rEPO. This is a qualitative method in which the detection criterion proposed were established by observing the variation or ratio concerning the intensity of the most plentiful bands in the exogenous