DETECTION OF RECOMBINANT HUMAN ERYTHROPOIETIN AND ANALOGUES THROUGH IMMUNORECOGNITION AND *N*-GLYCOLYL-NEURAMINIC ACID IDENTIFICATION

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Als meus pares,
a la meva germana,
a l'Esther,
a en Marc.

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Es busca bioquímic per realitzar la tesis doctoral en el projecte de recerca "Rapid screening (and confirmatory) method for rhEPO and NESP based on immunorecognition of its exogenous N-glycolylneuraminic acid content".
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Abbreviations

aa	amino acid
ACN	acetonitril
Asn	asparagine
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
BRP	Biological Reference Preparation
Dynepo	epoetin delta
DNA	desoxyribonucleic acid
cDNA	complementary desoxyribonucleic acid
CERA	Continuous Erythropoietin Receptor Activator (pegilated Epoetin beta)
Lys	lysine
CHO	Chineses Hamster Ovary
Cys	cysteine
DMB	1,2-diamino-4,5-methylenedioxybenzene
DTT	dithiothreitol
EIC	extracted ion chromatogram
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPO	erythropoietin
FA	formic acid
FBS	Fetal Bovine Serum
Gal	galactose
GlcNAc	*N*-acetylgalactosamine
HAT medium	Hypoxanthine-Aminopterin-Thymidine medium
Hb	haemoglobin
hEPO	human erythropoietin
HIF-1	hypoxia inducible factor-1
HPLC	high performance liquid chromatography
HPLC-Chip	nano-flow high performance liquid chormatography
HQC	high quality control
IAC	immunoaffinity column
IAP	immunoaffinity plate
Ig	immunoglobulin
IEF	isoelectric focusing
IOC	International Olympic Committee
JAK2	janus kinase 2
KLH	keyhole limpet hemocyanin
LOD	detection limit
LQC	low quality control
MAIIA	Membrane Assisted Isoform ImmunoAssay
MAPK	mitogen-activated protein kinase
MRM	multiple reaction monitoring
MS	mass spectrometry
Mw	molecular weight
mRNA	messenger ribonucleic acid

m/z	mass to charge ratio
NESP	novel erythropoietin – stimulating protein (darbepoetin – α)
Neu5Ac	*N*-acetylneuraminic acid
Neu5Ac-OVA	Neu5Ac-Gal-GlcNac-spacer-OVA
Neu5Gc	*N*-glycolylneuraminic acid
Neu5Gc-KLH	Neu5Gc-Gal-GlcNac-spacer-KLH
Neu5Gc-OVA	Neu5Gc-Gal-GlcNac-spacer-OVA
NIBSC	National Institute for Biological Standards & Control
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PI	phosphatidylinositol
PVDF	polyvinylidene difluoride membrane
PVP	polyvinylpyrrolidone
ret	reticulocites
rhEPO	recombinant huma erythropoietin
RP	reverse phase
RSD	relative standard deviation
SDS	sodium dodesil sulphate
Ser	serine
SEP	synthetic erythropoiesis protein
shEPO	serum human erythropoietin
sTFr	soluble transferrin receptor
std	standard
TFA	trifluoroacetic acid
uEPO	urinary erythropoietin
uhEPO	urinary human erythropoietin
WADA	World Anti-Doping Agency
WB	western blot
WGA	wheat germ agglutinin
[$^{13}C_3$]Neu5Ac	*N*-Acetyl-D-neuraminic acid-1,2,3-$^{13}C_3$

Abstract

Erythropoietin (EPO) is a glycoprotein hormone, the molecule comprises a single polypeptide chain of 165 aminoacids with two disulfide bonds, 1 O-linked (Ser-126), and 3 N-linked (Asn-24, 38, 83) glycans representing about 40 % of the total mass (~30 kDa). It is secreted primarily by adult kidneys in response to tissue hypoxia and it is involved in the maturation and ultimately regulation of the level of red blood cells. The recombinant analogue (rhEPO), available since 1989 has found widespread use in the treatment of different diseases. Besides, rhEPO is illicitly used by athletes to boost the delivery of oxygen to the tissue and enhance performance in endurance sports. The most important recombinant EPOs and analogues used in sport are rhEPOs, NESP and CERA. Current tests to differentiate between endogenous EPO and its recombinant analogues are based on differences in their bioelectric focussing (IEF) profiles and on differences in their molecular weight (SDS-PAGE). In this study, different methods to facilitate the detection of recombinant EPOs and analogues in antidoping control have been developed: A plasmatic EPO immunopurification method; a new screening method based on immunoaffinity techniques to detect the abuse of recombinant erythropietins in urine; and a liquid chromatography-mass spectrometry method that allows to detect the unambiguous differing structure between exogenous EPOs and endogenous, the N-glycolyl-neuraminic acid.

Resum

La eritropoetina (EPO) és una hormona glicoproteica formada per una cadena peptídica de 165 aminoàcids que conté dos ponts disulfur, un O-glicà (Ser-126) i tres N-glicans (Asn-24, 38, 83) que representen al voltant d'un 40% de la seva massa molar (~ 30kDa). Es produeix principalment en el ronyó, en resposta a la reducció d'oxigen en el teixits, i estimula l'eritropoesi a la medul·la òssia. La EPO recombinant (rhEPO) s'administra com a fàrmac pel tractament de diferents malalties. També s'ha observat la seva utilització en esportistes amb l'objectiu d'augmentar el nivells d'oxigen als teixits i així incrementar el seu rendiment. Les EPOs recombinants i anàlegs més enmprades en l'esport són les rhEPOs, NESP i CERA. Els mètodes que s'utilitzen per diferenciar la eritropoetina orinaria endògena de l'exògena estan basats en diferencies dels seus perfils isoelectroforètics (IEF) o en els seus pesos moleculars (SDS-PAGE). El problema d'aquests mètodes és que són llargs, costosos i només poden utilitzar la orina com a matriu biològica. En aquest estudi, s'ha dut a terme el desenvolupament dels següents mètodes que faciliten la detecció d'EPOs recombinants y anàlegs en el control antidopatge: Un mètode d'immunopurificació d'EPO en plasma; un mètode d'screening ràpid basat en tècniques d'immunoafinitat per detectar l'abús d' eritropoietines recombinants en orina; i un mètode de cromatografia liquida acoblada a espectrometria de masses que permet detectar una clara diferencia estructural entre la majoria de les EPOs exògenes i la endògena, el *N*-glicolil-neuraminic àcid.

Prologue

This year, 2011, mark twenty-two years since the production of recombinant human erythropoietin, or as it is commonly known rhEPO. Nowadays, rhEPOs and its analogues are one of the most important therapeutic agents for the treatment of chronic renal failure and malignancies. However, the availability of the rhEPO and its benefits for sportsmen, inducing a greater power and resistance, has increased the risk of its illegal use in sports.

Due to the existence of new technologies, amazing advancements have been made during the last decade on the detection of these doping agents in urine. However, there is still much to be done, particularly in the detection of these doping agents in other biological fluids as serum or plasma, and in the development of fast and cheap screening methods that allows detecting the abuse of these substances in all samples collected.. This thesis aims at making a contribution in this field.

This manuscript is structured in six main chapters, each containing several sub-chapters. The first chapter includes the *Introduction*, covering background information on Erythropoietin, its biological function and fate as well as its misuse in sport (doping) and the analytical approaches developed to detect its abuse. The second chapter comprises the general and specific *Objectives* of the work. The third chapter contains the *Results* obtained, embedding the corresponding publications, when available, or describing other non-published results together with a brief introduction followed by materials and methods. In this chapter, different tools to make easy the detection of rhEPO abuse such as a plasmatic EPO immunopurification method, a rapid screening method for rhEPOs and analogues based in immunorecognition, and a chip LC/MS/MS method

for the detection of *N*-glycolyl-neuraminic acid (Neu5Gc) are described. The results evidence the benefits of using these complementary methods to the official IEF method for detecting abuse of rhEPOs. The fourth chapter contains an overall *Discussion* of all results presented. The fifth chapter lists the *Conclusions* of the thesis. References are included as a last chapter.

Part of the work described in this thesis had the contribution of a number of people. Dr. Carlo Unverzagt from the University of Bayreuth synthesized the specific trisaccharides used for the production of monoclonal antibodies against Neu5Gc, Dr. Esther Llop developed a HPLC-FLD method for the detection of Neu5Gc in rhEPOs, and Dr. Ricardo Gutiérrez-Gallego, Dr. Carme de Bolós, Dr. Jordi Segura and specially Dr. J. Antonio Pascual contributed in the design and discussion of the work.

CONTENTS

1. Introduction

1.1. Erythropoietin

1.1.1. Function and productions sites

Human erythropoietin (hEPO) is a glycoprotein produced in response to the oxygen tension of the blood. It is mainly produced by the peritubular fibroblast-like cells located in the cortex of the kidney in adults and by hepatocytes during the fetal stage. EPO circulates to the bone morrow where it stimulates proliferation and differentiation of the red blood cell progenitors, leading to more red blood cells and increased oxigen-carrying capacity.

Recent studies have shown that EPO is a pleiotropic hormone. In addition to the kidney also liver, spleen, lung, bone marrow and brain were shown to express EPO mRNA [1]. Brain-derived EPO, which is unlikely to enter the general circulation in significant amounts because of the blood-brain barrier [2], is thought to act as a paracrine neuroprotective factor.

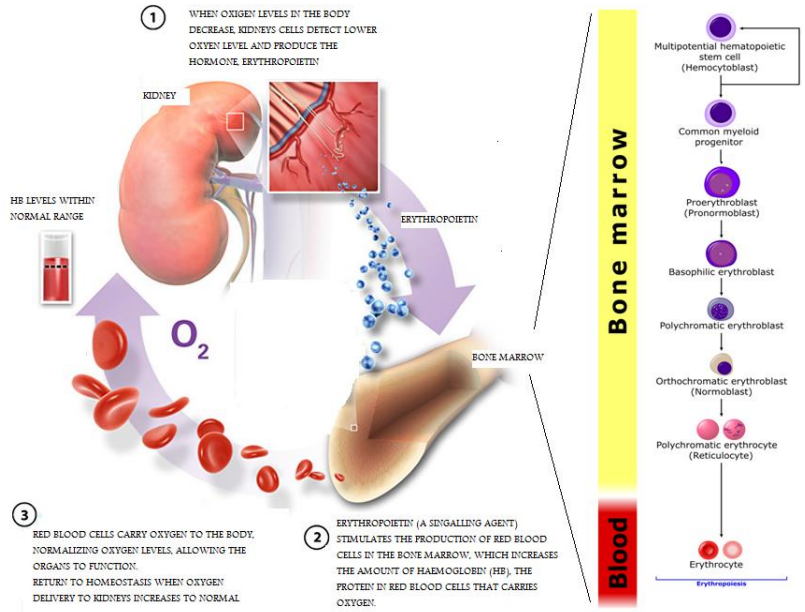

Figure 1. Scheme of human erythropiesis (right) and feed-back mechanism for regulating erythropoietin (left).

1.1.2. Regulation of the Erythropoietin Gene

Tissue hypoxia is the main stimulus of EPO production and secretion. EPO is not only produced when oxygen capacity of the blood decreases (hypoxia), but also when arterial pO2 decreases or when the oxygen affinity of the blood increases.

In most tissues, including kidney, liver, uterus and other organs like brain, the EPO gene expression is induced by hypoxia-inducible transcription factors (HIFs). The principal representative of the HIF-family is HIF-1, a heterodimeric protein composed of an alpha subunit (HIF-1alpha, 120 kDa) and a beta subunit (HIF-1Beta, 91-94 kDa) that is activated by a variety of stressors, including hypoxia [3]. However there are other transcription factors which can modulate EPO gene transcription.

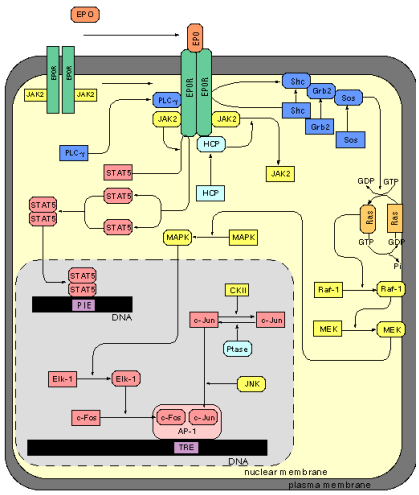

Figure 2. Scheme of EPO signalling pathways. The signalling cascade results in survival, proliferation and differentiation of erythrocytic progenitors. [From http://www.grt.kyushu-u.ac.jp/spad/pathway/epo.html]

EPO-receptor binding induces a conformational change and a tighter connection of the two receptor molecules [4, 5]. As a result, two Janus kinase 2 (JAK2) tyrosine kinase, which are in contact with the cytoplasmic

region of the EPO receptor, are activated. Then, several tyrosine residues of the EPO receptor are phosphorylated and exhibit docking sites for signalling proteins containing SRC homology 2 (SH2) domains. As a result, several signal transduction pathways are channelled, including phosphatidyl-inositol 3-kinasa (PI-3K/Akt), JAK2, STAT 5, MAP kinases and protein kinase C. However, the specific roles of the various enzymes and transcriptional cofactors are only beginning to be understood. The effect of EPO is terminated by the action of the hemopoietic cell phosphatase (HCP) which catalyses JAK2 de-phosphorylation. Apparently, the EPO/EPO-receptor complex is internalized following de-phosphorylation of the receptor.

1.1.3. Metabolism

EPO is distributed largely intravascularly and it is cleared from circulation with a fairly short half-life. However, the mechanisms responsible for clearance of EPO from the circulation are still under investigation. Different studies suggested that to a minor degree, EPO may be cleared by the kidneys following glomerular filtration (by the galactose receptor), once it is desialylated by action of tissue and blood sialidases in the liver [6]. However, there is evidence to assume that EPO is mainly removed from circulation by uptake into erythrocytic and other cells possessing the EPO receptor.

1.1.4. Biochemistry

The human EPO gene is located on the long arm of chromosome 7 (q11-q22). It contains five exons, which encode a 193 amino acid pro-hormone including a 27 aa signaling peptide, and four introns. The 166-amino acid protein has a molecular weight of 19,398 Da [7].

The resulting glycoprotein hormone has a molecular mass of 30.4 kDa. The peptide core of mature EPO consists of a single 165 aa polypeptide chain (the signaling peptide is cleaved prior to secretion and the circulating human EPO lacks the carboxy-terminal arginine). It has two disulfide bonds (Cys-7 – Cys-161 and Cys-29 – Cys-33) and four glycosylation sites that provide three N-linked (Asn-24, 38, 83) and one O-linked (Ser-126) oligosaccharide chains. The resulting carbohydrate content accounts for roughly 40 % of the total molecular mass of the glycoprotein.

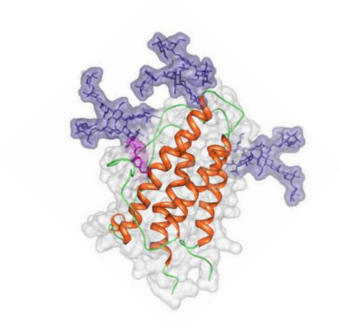

Figure 3. Model of the three dimensional structure of erythropoietin. The four α-helices are in orange, loops between helices are depicted in green. The 3 N- and 1 O-glycosylation sites are indicated in violet and pink respectively. [From www.glycam.com]

The N-glycosilation is essential for the in vivo biological activity of EPO, especially, the terminal sialic acid residues of these glycans [8, 9]. When these residues are removed from EPO (e.g. with sialidase), the resulting molecules have an increased activity in vitro, but less activity in vivo, presumably due to removal from circulating by the asialoglycoprotein receptor in the liver [10]. Why glycosylation increases EPO's in vivo half-live is not fully understood, but it has been proposed that enlarging the Stokes' radius or "hygrodynamic size" of the molecule in some way reduces its clearance.

1.1.5. Recombinant erythropoietins, analogues and mimetics

A) Recombinant Human erythropoietin (epoetin)

In 1977 small amounts of human erythropoietin from the urine of patients with aplastic anaemia were purified. Based on the limited peptide sequence information obtained from this purified material, the gene for human erythropoietin was then isolated and cloned in 1983 [11]. The use of genetic engineering techniques finally allowed the large-scale production of recombinant human erythropoietin in a suitable mammalian cell line. It has become one of the most important biotechnology products as it has provided new therapeutic solutions for a variety of diseases and oxygen-deficiency states (e.g. renal anaemia and anaemia of cancer) [12].
Recombinant human erythropoietin has been produced using different mammalian cell lines as Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells or even human cells (HT1080). All this recombinant EPOs have the same amino acid sequence but the different preparations show differences in their degree of glycosilation as well as in their glycan composition and/or structure due to differences on the cell lines used to express the proteins and the purification strategies used. Different formulations of recombinant EPO have been developed both in academia [13, 14] and by pharmaceutical industries. Also with the expiration of patents for epoetins, new versions of these products and generics appeared in the market.

B) Darbepoetin alfa or Novel Erythropoiesis Stimulating Protein (NESP)

Darbepoetin alpha was created using site-directed mutagenesis to insert an additional two additional N-linked glycosylation chains into the protein (at Asn-30 and Asn-88). The strategy required the substitution of a total of five aminoacids [15]. As a consequence, NESP has an increased molecular

mass (37.1 kDa) and an increased proportion of carbohydrates (51 %) as compared to the Epoetins (41 %).

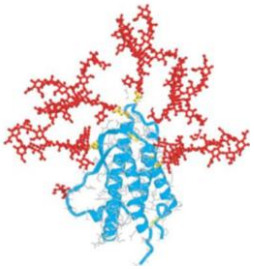

Figure 4. Model of the three dimensional structure of NESP. The four α-helices are in blue and the 5 N- and 1 O-glycosylation sites are indicated in red. [Adapted from M.R. Wormald, R.A. Dwek (Oxford Glycobiology Institute) and P.M. Rudd (NIBRT)].

Owing to the additional sialic acid content, NESP has a slower serum clearance and, therefore, a longer half-life than the eopetins [16]. The terminal half-life of i.v. administered Darbepoetin-alpha is three- to fourfold longer than that Epoetin-alpha and –beta (25.3 h vs 8.5 h), thus, affecting the biochemical and biological properties of NESP.

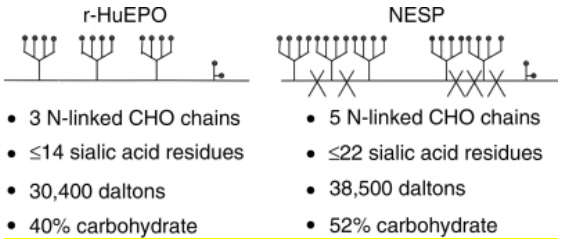

Figure 5. Comparison of the structure of darbepoetin alfa and rHuEPO.
The "X"s in darbepoetin alfa represent the five amino acid exchange sites that were required to allow the attachment of two extra N-linked carbohydrate chains. [From Macdougall, 2002, [17]]

C) Continuous Erythropoietin Receptor Activator (CERA)

The continuous erythropoietin receptor activator is a pegylated Epoetin beta. It was created by integrating a single 30 kDa methoxy-polyethylene glycol polymer chain into the erythropoeitin molecule. This integration was achieved through amide bonds form by the N-terminal amino group as well as the ε-amino group of lysines (predominantly Lys-52 or Lys-45), with a single succinimidyl butanoic acid linker [18]. The resulting molecular mass is about 60 kDa, twice the epoetin's size.

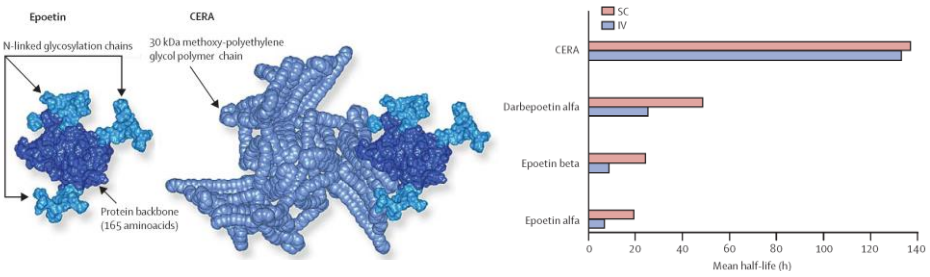

Figure 6. Comparison of epoetin and CERA structures (right) and representation of mean half-lives of CERA, darbepoetin alfa (NESP), epoetin beta and epoetin alfa (letf). From Macdougall, 2006, [19].

CERA has an even longer half-life than Epoetins and Darbepoetin-alpha in circulation, about 130 or 140 hours. The hypothesis is that the binding of CERA to its receptor is too brief to allow internalization of the molecule. Therefore the repeated binding, stimulation and dissociation lead to prolonged activity in vivo and extended elimination half-life [20].

D) Synthetic erythropoiesis protein (SEP)

Synthetic erythropoiesis protein is another erythropoietic polymer. Using solid phase peptide synthesis and branched precision polymer constructs, a 51 kDa protein-polymer construct has been made containing a 166-

amino-acids polypeptide chain (similar to the sequence of EPO) and two covalently attached polymer moieties.

The resulting polymer stimulates erythropoiesis through activation of the erythropoietin receptor. It was reported that SEP had superior duration of action in vivo and a longer circulation lifetime than EPO [21].

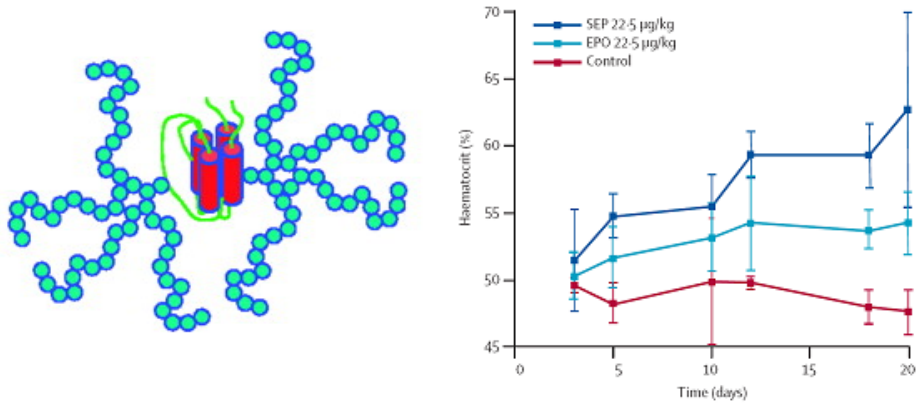

Figure 7. Model of the three dimensional structure of SEP (left). [From Kochendoerfer, 2003, [21]]. Haemopoietic activity SEP and EPO given once weekly to normal mice (right). [From Macdougall, 2006, [19].

E) Erythropoietin-mimetic peptides and nonpeptides

The EPO-mimetics are small molecules capable of dimerizing the EPO receptor and act in the same way as EPO. There are two groups of EPO-mimetics, the peptides and the nonpeptides.

EPO-mimetic peptides were obtained from screening random peptide-phage libraries in the search for an agonist peptide [22, 23]. Most of these molecules possess shorter in vivo half-lifes than EPO. However, Hematide, an EPO-mimetic peptide attached to polyethylene glycol, has a long circulating half-life and extended duration of erythropoietic effect [24].

Small molecules from non-peptide libraries have also been screened to identify a molecule able to bind to the erythropoietin receptor. Several compounds had been selected, but their EPO receptor affinity and biological activity were much lower than those of EPO [25].

F) Erythropoietin oligomers and fusion protein

Another possibility for the anaemia treatment is derived from a cDNA encoding fusion protein of two complete human erythropoietin domains linked by a 17-aminoacid flexible peptide. It seems that a single subcutaneous dose of EPO-EPO fusion proteins resulted in a significant increase in hematocrit within seven days, whereas administration of an equivalent dose of conventional recombinant EPO did not produce any effect.

Fusion proteins of EPO with hematopoietic growth factors have also been described [26-28]. These fusion proteins exhibited enhanced erythropoietic activity in vitro as compared to recombinant EPO alone.

Finally, another interesting possibility is a fusion protein of EPO with the Fc portion of immunoglobulin (Ig) (e.g. CTNO 528; Centocor®). The Fc portion of Ig imparts the prolonged in vivo half-life characteristic of Ig [29].

G) Erythropoietin gene therapy

Another approach intended to replace injections withrecombinant EPO is gene therapy. There have been numerous methods studied [30]. They include direct injection of EPO expression plasmids into muscle or liver, introduction of the EPO gene using various viral vectors, and implantable capsules containing cells expressing the EPO gene.

In 2002, a British pharmaceutical company (Oxford BioMedica) developed *Repoxygen* as a treatment for severe anemia. Repoxygen is based

on an experimental virus designed to insert a therapeutic gene into a person's DNA. Repoxygen is the tradename for a type of gene therapy that induces controlled release of EPO in response to low oxygen concentration. After its development in mice, it is still in preclinical development. This approach to EPO therapy will require many years of development [31]. None of these products has as yet arrived to the clinical use.

1.1.6. Erythropoietin and sport

The ability to carry sufficient oxygen and nutritional substances to the muscles represents the major limit to intensity and length of physical effort. Despite the very effective homeostatic mechanism humans possess, oxygen resources are rapidly consumed during intense and extended physical activities resulting in a decrease of muscular function. As the largest part of oxygen in blood is normally carried by red blood cells, a substantial increase in erythrocyte count induces a greater power and resistance [32].

It is well known by athletes that increasing the oxygen carrying capacity of the blood that accompanies red cell mass improves endurance. Therefore, blood transfusions were and there may still be used. The expansion of blood volume is prohibited in sport and is considered as "blood doping". Already in 1987, the International Olympic Committee (IOC) banned blood doping.

Following the cloning of the EPO gene in 1985, the rhEPO was available as a drug for the clinical treatment. The result of the administration of rhEPO is basically identical to transfusion. However, some adverse effects of the latter, e.g. as allergic reactions or haemolytic crisis, are virtually absent. For all these reasons the treatment with rhEPO in sport had an enormously diffusion. After EPO became available, numerous

unexplained deaths were noted among competitive cyclists, believed to involve EPO use.

The excessive use of EPO is associated with serious adverse side-effects, including hypertension, headaches, and a n increased rate of thrombotic events as a resutld of an EPO-induced rise in the hematocrit and thickening of the blood [33]. In addition, EPO withdrawal could be implanted in neocytolysis, that is, the hemolysisi of young red blood cells in the presence of increased hematocrit. Ultimately, EPO abuse could cause death [34]. For all these reasons, in 1990, the IOC added EPO to its "List of Prohibited Substances" [35].

In 1998, as a direct result of the apparently widespread use of recombinant EPO by cyclists, particularly during the 1998 Tour de France, the IOC encouraged the creation of the World Anti-Doping Agency (WADA). This Agency, finally created in 1999, has the mission to *"promote, coordinate and monitor the fight against doping in sport in all its forms"*. WADA created the World Anti-Doping Code and its associated International Standards to harmonise the applicable rules. One of those standards was the Prohibited List, revised annually, where EPO was included as a doping agent [35].

In the last ten years, there have been various rumours and scandals related to rhEPO abuse. The most important one was the called "Operación Puerto" in 2006. This judicial operation unveiled the link between several elite cyclists and other sportsmen with blood-doping practices, and seized multiple bags of blood products for reinfusion. Many examples of EPO abuse by elite athletes have been and are still being reported.

1.2. Procedures for monitoring recombinant erythropoietin and analogues in doping control

The detection of rhEPO and analogues has shown to be very challenging due to different factors. Firstly because they are virtually identical to their endogenous counterparts [36-38]. The only alleged differences so far seem to be located in their carbohydrates [39, 40]. Secondly because they are present in urine at very low concentrations (ca.< 1 pM)[41]. Thirdly because, as glycoproteins, they are not pure single chemical entities but composed by a plethora of so-called isoforms. Thus each "detectable" isoform is present in much lower concentrations (ca. < 10 fM). However, different doping tests have been developed and used in the last years. These doping tests are classified as direct or indirect [42]. A direct test identifies the doping substance, either chemically or biochemically. An indirect test measures biologic markers that accompany the use of the substance without necessarily directly identifying it.

1.2.1. Indirect methods

Five different hematopoietic parameters were chosen as the most clearly affected by the administration of rhEPO: serum EPO concentration, serum soluble transferrin receptor concentration (sTFr), hematocrit, percentage of reticulocytes (young red blood cells), and a percentage macrocytes. After statistical evalution, two discriminant models were built: The "ON" model, fitting the data during treatment or shortly after, and the "OFF" model, fitting the data weeks after stopping treatment [43]. When the models were developed, a cut off value for each score had to be defined to identify samples as "presumptive positive". With the aim to have a simpler and more sensitive test, a so called second-generation test was develop ped, parameters could be reduced to only haemoglobin and

EPO concentration in serum for the "ON" model and hemoglobin concentration plus reticulocyte percentatge for the "OFF" model.

ON model

On score = Hb + 9.74 In (EPO) or Hb + 6.62 In (EPO) + 19.4 In (sTfr).

Off model

Off score = Hb – 60 (ret %) 1/2 or Hb – 50 (ret %) 1/2 – 7 In (EPO)

This second-generation model seemed to be more sensitive when low doses of rhEPO were used. However, it still had two drawbacks: it requires the use of blood, which is not the regular specimen obtained from athletes, and it makes counter analysis impossible because of the instability of the parameters measured in whole blood. Nevertheless, the method is fast and relatively cheap so that it can be used for screening/targeting purposes.

1.2.2. Direct methods

Different direct methods have been published since 2000; the IEF method, the SDS-PAGE method and EPO WGA MAIIA method. The only method currently accepted by the World Anti-Doping Agency to detect abuse of rhEPO and analogues, is the isoelectric focusing (IEF) described by Lasne et al.

The IEF method has been implemented by the antidoping laboratories, as a routine test. However, in some cases, the SDS-PAGE method is also needed to obtain additional evidence when routine results are inconclusive.

A) Isoelectric focusing (IEF)

The IEF method, published in 2000 by Lasne et al. [44, 45] is based on the differences observed in the charge of the isoforms of the recombinantly produced EPO (rhEPO) with respect to the endogenously produced urinary EPO (uhEPO).

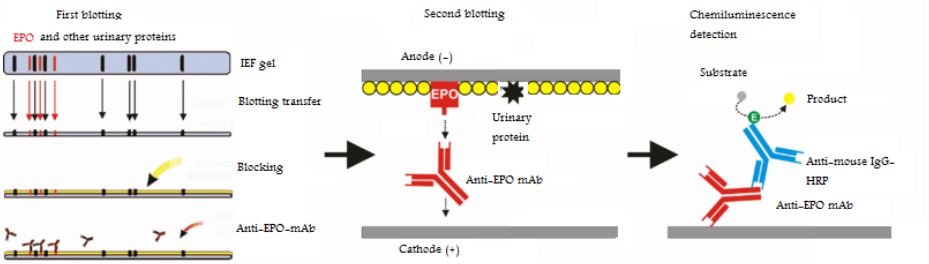

Figure 8. Description of the different steps of the IEF method.

IEF gel and first blooting (left), second blotting (middle) and chemiluminescence detection (right).

The method described by Lasne et al. uses isoelectric focusing in gel. After separation according their isoelectric point (pI), proteins are transferred from the gel onto a membrane (blotting), where both recombinant and natural EPO are targeted by a monoclonal antibody raised againts EPO. Then, the antibody is transferred to a second membrane (double blotting) where it is addressed by a second, biotin-labelled, anti-species polyclonal antibody. Finally, spots containing biotin are recognized by streptaviding bound to horseradish peroxidase. In the end, a peroxidase-labelled spot is obtained. When chemiluminescent reagents and hydrogen peroxide get in contact with the proxidase-labelled spots, light is generated. This light can be detected with extremely high sensitivity. As a result, an image is obtained containing the spots where EPO was recognized.

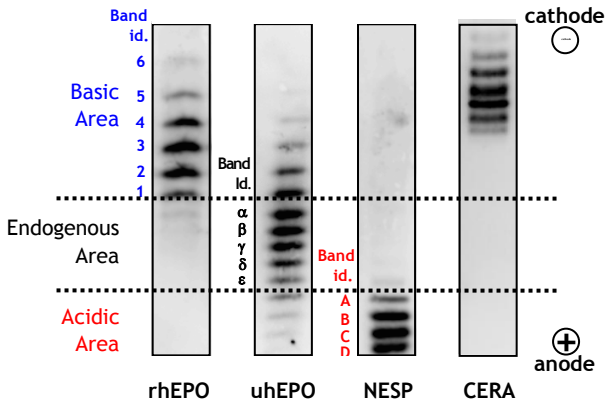

Figure 9. Images corresponding to the analysis of the recombinant materials (rhEPO, NESP and CERA) as well as human urinary EPO (uhEPO) using isoelectric focusing (IEF), double blotting and chemiluminescent detection.

Using this method, it can be observed that endogenous urinary EPO shows to a pattern of spots different (more acidic) than the one obtained for rhEPO.

NESP also shows a different profile, as it is a hyperglycosylated version of EPO. Its four major bands appear as a cluster in the most acidic region of the gel. Conversely, CERA shows a very characteristic pattern of at least 6 bands in the basic area, above rhEPO [46]. The SEP show a single band, isoelectirc point is approximately 5, in IEF.

Unfortunately those differences do not allow an absolute identification of the presence of the recombinant species since endogenous and recombinant seem to show just differences in the proportion in which each isoform is expressed rather than showing specific new ones, probably except CERA where some of the bands appeard interspersed amongst rhEPO bands [46]. This has forced the use of different evaluation criteria [47], not always reproducible or easy to apply to identify rhEPO in the presence of uEPO.

Furthermore, the overall method is not amenable for screening purposes since it is expensive, labour intensive and very time consuming (up to three days for a single gel analysis).

B) SDS-PAGE

In 2007, Kohler et al. were the first to publish on the potential use of SDS-PAGE, which separates proteins according to their apparent molecular mass, to discriminate between recombinant and endogenous urinary EPO [48].

Briefly, 20mL of urine are concentrated by filtration and then immunopurified by an anti-EPO enzyme-linked immunosorbent assay (ELISA). Then proteins are reduced with DTT and applied to an SDS-PAGE gel where they are separated. Finally the procedure continues as in the IEF method with a blotting or double blotting and chemiluminescent detection.

NESP and recombinant rat EPO were taken as internal standards to calculate relative mobility values. Their behaviour in the SDS-PAGE is different from the other erythropoietins as NESP has two additional N-glycosilation sites, so it has a higher molecular mass (ca. 37,400 Da) whereas recombinant rat EPO (produced in insect cells) has a lower molecular mass of approximately 21,300 Da. Epoetin alpha, beta and delta yield similar molecular masses between 29,000 and 30,000 Da. SEP migrates as a single sharp band with an apparent molecular mass of 73 kDa while EPO-dimer. The fusion protein, migrates at 76 kDa, slightly greater than twice the average for rhEPO. Indeed uhEPO and serum human EPO (shEPO) showed a slightly lower molecular mass compared to most rhEPOs (such as epoetins alpha, beta, and delta). Therefore, this slight difference in migration can be used as additional evidence to differentiate between endoengous and exogenous erythropoietins.

The method may be useful as additional confirmatory evidence, complementary to the established IEF assay.

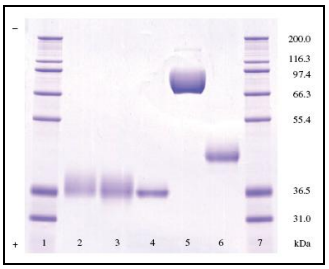

Figure 10. Image corresponding to the analysis of the recombinant materials as well as endogenous urinary EPO using SDS-PAGE, western blotting and chemiluminescent detection. 1. Molecular weigth, 2. Erypo, 3. Neorecormon, 4. Dynepo, 5. CERA, 6. NESP and 7. Molecular weight. [From C. Reichel, 2009, [49]]

In 2009, Reichel et al. [49] pointed out some of the specific benefits of the SDS-PAGE method and in particular for the identification of Epoetin delta (Dynepo, produced in a human cell line). Dynepo shows an IEF profile shifted towards more acidic pI values (endogenous area). That feature makes its identification very difficult, defeating the criteria established for other rhEPOs. In SDS-PAGE on the contrary, Epoetin delta shows a sharp band, unusual when comapred to epoetin alpha, beta, omega, darbepoetin alpha, PEGylated epoetin beta (MIRCERA), biosimilars, and even human urinary and serum EPO (Figure 10). Due to this very characteristic band shape Dynepo appeared to be much better detected by SDS-PAGE. Furthermore, SDS-PAGE also revealed additional information to discriminate the so called "active" (unstable) and atypical or effort-type IEF-profiles. As separation by apparent molecular mass was barely unaffected by those features, they could be distinguished from those genuinely affected by the abuse of recombinant EPO.

C) EPO WGA MAIIA method

In 2009, Lönnberg et al. developed the EPO WGA MAIIA method [50]. The method exploits the different affinity of a lectin, wheat germ agglutinin (WGA), for rhEPOs and uhEPO.

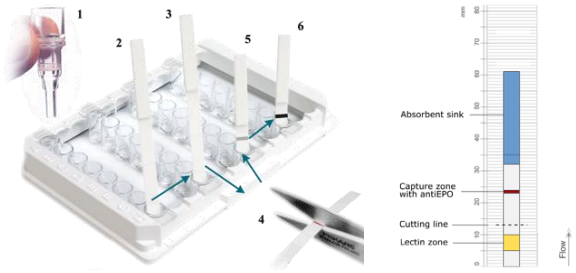

Figure 11. Description of the micro-column strip parts (right) and description of the procedure for EPO doping test using MAIIA kit (left). 1. EPO immunpurification, 2. Sample incubation, 3. Desorbation solution incubation, 4. Cutting 5. Carbon black nanostring anti-EPO incubation, 6. Washing.
[From http://www.maiiadiagnostics.com/research/epo_doping_test.htm].

Briefly, samples are immunopurified by anti-EPO affinity purification cartridge (also developed by MAIIA diagnostics), then the micro-column strip is placed in a well with 25 µl of immunopurified sample. All glycoproteins are trapped by the lectin ("lectin zone"). Then, the strip is moved to another well where the captured glycoproteins are displaced from the lectin zone by an *N*-acetyl-glucosamine containing buffer. Using the appropriate concentration, uhEPO is eluted slightly before than other rhEPOs and analogues. Finally EPOs are captured in a sharp zone containing a monoclonal anti-EPO antibody. ("capture zone") and revelaed with a secondary anti-EPO antibody labelled with carbon nanostrings. The balckness of the resulting band is measured usign a scanner. By exactly reproducing the displacement times a difference can be found in the quantification of EPO when the sample contains only

uhEPO or a mixture with rhEPO. Two quantifications are necessary to detect the presence of exogenous EPO, the total EPO content and the fraction eluted under appropriate conditions from the lectin zone.

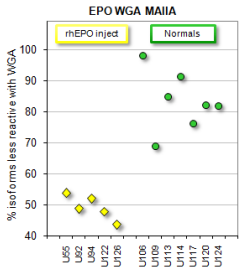

Figure 12: Results corresponding to the analysis of EPO from 12 urines (seven from healthy humans and five from humans injected with rhEPO) using the EPO WGA MAIIA test. The EPO WGA MAIIA distinguishes recombinant from endogenous EPO due to their differences in interaction with the WGA lectin. Endogenous isoforms interact less with WGA than recombinant ones.
[From http://www.maiiadiagnostics.com/research/epo_doping_test.htm]

D) rhEPO detection based on the presence of *N*-glycolyl-neuraminic acid. Another approach to differentiate recombinant EPO from endogenous EPO is the detection of *N*-glycolyl-neuraminic acid (Neu5Gc), a non-human sialic acid, in EPO. Sialic (or neuraminic) acids are the charged monosaccharides present in the outer terminal positions of the glycans attached to the protein backbone. They are, by their variable occurrence, the main responsible for the band profile displayed by glycoproteins in IEF. The most usual sialic acid present ubiquitously is *N*-acetyl-neuraminic acid (Neu5Ac). Other poly-acetylated ones (e.g. Neu5,9Ac2) are also frequent in much lower amounts [51, 52]. Neu5Gc is another sialic acid very frequent found in most mammals including our closest relatives, the great apes [53]. However, this sialic acid cannot be endogenously produced by humans since we lack the corresponding enzyme (CMP-Neu5Ac hydroxylase) [54, 55]. In 1990, Hokke [56]

described that the EPO produced in CHO cells consistently contained small amounts of Neu5Gc. And, in 1993, Nimtz [57] described the same finding for BHK cells, another extensively used cell line used for the expression of recombinant proteins. Hence, the Neu5Gc presence in human plasma or urine would constitute the proof or suspect of the abuse of some recombinant glycoproteins by athletes (rhEPO, recombinant human chorionic gonadotrophin, luteinizing hormone). However, the presence of minute amounts of Neu5Gc in human carcinomas [58] and fetal tissues has been demonstrated. It has also been described that humans may absorb small quantities of Neu5Gc from dietary sources and metabolically incorporate them into certain cell types [53]. A similar "contamination" by Neu5Gc apparently could occur also in the biotechnology industry, arising from the use of animal cells, ser or other products during manufacture.

Sialic acids from glycoproteins have been traditionally determined by labelling with 1,2-diamino-4,5-methylenedioxybenzen (DMB) followed by conventional high performance liquid chromatography (HPLC) with fluorescent detection [59-61].

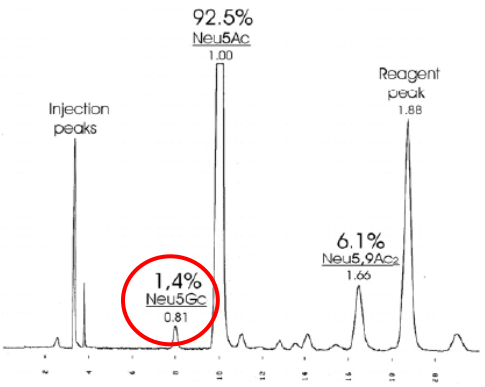

Figure 13. Analysis of the sialic acid content of rhEPO by RP-HPLC with fluorescence detection of the corresponding DMB derivatives.

In the last years, efforts have been made in our group to improve the sensitivity of these methods by using a capillary hplc-fluorescence detection system. This technique allowed us to arrive to a limit of detection of 6 fmol (~1.5 pg) for Neu5Gc (signal-to-noise ratio =3).

The use of HPLC coupled to mass spectrometry, on the other hand, has the advantage of improving selectivity and providing structural information, thus allowing identification of the sialic acid species detected. In the last years, several works have been published addressing this issue [62, 63]. However, the major drawback was that the sensitivity achieved by mass spectrometry was much lower than the one achieved by flurescence detection. In order to achieved a comparable limit of detection, in 2007 Noritaka et al. used a nano-flow liquid chromatography coupled to Fourier transformation ion cycrotron resonance mass spectrometry (nanoLC/FTMS) [62] and arrived to 7,8 fmols of Neu5Gc.

The sensitivity of all this methods is not sufficient to detect Neu5Gc in the concentrations that are present in human urine or blood. For this reason, an alternative method for Neu5Gc detection in biological fluids is required. An ELISA test or another amplifiable immunodetection method using antibodies against Neu5Gc could be the alternative method. The only commercially available anti-Neu5Gc antibody (from GC-free Inc., San Diego, California) does not have the required sensitivity either. Therefore, the development of a monoclonal antibody against Neu5Gc in glycoprotein is necessary. Although sugars are not considered very immunogenic, different antibodies have already been described able to recognize Neu5Gc (while not Neu5Ac) present in, for example gangliosides [64, 65]. So using the appropriate material it should be

possible to generate monoclonal antibodies able to pick those non-human tags present in the recombinant materials.

1.3. Erythropoietin purification

Erythropoietin purification is required for two different reasons: the first one is that pure EPO is required for its characterisation. The second reason, and the most important one for our work, is that all direct methods explained before need a prior immunopurification step for EPO analysis.

However, the isolation and purification of naturally occurring EPO is a difficult task given the large amount of starting material needed and the optimisation of the assay required. It was clear from early studies that EPO was not stored in great quantities in any organ of the body, so there were no clusters of EPO-producing cells that could be isolated readily from which substantial amounts of hormone could be purified [66]. Potentially sources of naturally occurring EPO included the urine or plasma of anaemic large animals, including humans [67], various organs such as the kidney, and cell lines derived from tumours such as renal tumours that spontaneously produced EPO [68].

Different immunaffinity techniques had been developed with the aim of purifying EPO from urine or blood since 1970.

1.3.1. Purification of urinary human EPO

An interesting approach to the isolation of EPO in the late 1970s was taken by Spivak et al. [69]. Immobilinzing WGA on agarose allowed an 8- to 100-fold purification of human urinary EPO (uhEPO) with recoveries of reater than 40 %. However, homogenicity was not achieved.

In 1977, Miyake et al. described a seven steps procedure that yielded highly purified uhEPO [67]. Remarkably, the starting material was

approximately 250 litters of urine collected in Japan from patients with aplastic anemia. The urinary protein was isolated and liophilised. A lot of work was needed but finally, a purification scheme was developed. It resulted in an EPO preparation with potency of 70,400 U/mg with a 21% yield. Interestingly, two pure fractions were obtained that exhibited slightly different motilities when subjected to gel electrophoresis at pH 9. An asialo EPO form was also identified. In this case, the apparent Mw of native EPO determined by SDS-PAGE was 39 kDa. Another purification method for urinary EPO was reported by Sasaki et al. [70] and consisted of preparation of an immunoaffinity column by coupling a monoclonal antibody against EPO to agaroses [71, 72]. Approximately 6 mg of EPO were isolated from around 700 litters of human urine. They reported at specific activity of 81,600 U/mg. Some heterogeneity was observed by SDS-PAGE and western-blot, being presumably due to partial deglycosylation. They also reported an N-terminal amino acid sequence of 30 aa, which differed in three positions from the N-terminal ovine EPO sequence disclosed by Goldwasser et al [73].

In the 2000s, different purification methods for uhEPO were reported. All of them were intended to be a first step prior to the analysis by the available methods to detect EPO in doping control, as IEF or SDS-PAGE. In 2002, a lectin immunoaffinity column (IAC) has been proposed for further cleanup of the samples in between or after the two ultrafiltrations steps of the IEF method [74]. But as a kind of ligand specific for carbohydrates, lectins will interact with other glycoproteins with similar structures which might influence the following detection. Another IAC, using polyclonal anti-EPO antibodies, to purify urinary EPO was reported by Mi. et al. [75]. The IAC was generated by covalent immobilization of anti-EPO antibodies on Sepharosa 4B support. The EPO-binding capacity of the IAC was found to be about 2 μg per 1.5 mL

of gel and the recoveries were between 78 and 86 % for rhEPO at low concentrations of 7.8, 10 and 120 IU/L.

Finally, the last method to purify uhEPO EPO was described by Kohler et al. [48]. Pre-concentrated urines from healthy subjects were incubated in an anti-EPO ELISA well plate. EPO was eluted with lithium dodecyl sulphate (LDS). This immunopurification allowed analysing EPO by the SDS-PAGE method. Despite the publication of these purification methods for analytical purposes, pure urinary human EPO in sufficient amounts to allow structure elucidation remains an unsolved task.

1.3.2. Purification of serum or plasma human EPO

After several years of work to purify EPO form the plasma of anemic sheep, Goldwasser and Kung reported the isolation of sub-milligram amounts of apparently pure material. Because the EPO amount available was so small, data obtained were limited. Only the apparent molecular mass was estimated by SDS-PAGE (46 kDa.) and sedimentation coefficient (4.6s) were reported.

In 1998, Skibeli et al. described a method to isolate EPO from sera [76] obtained from anemic patients using magnetic beads coated with a human EPO specific antibody. The method was later used for the isolation of EPO from the serum of anemic human donors; the main purpose was the study of the biochemical properties of human serum EPO (shEPO) [77]. Authors described that shEPO contained only mono-, di-, an tri-acidic oligosaccharides, lacking the tetra-acidic oligosaccharides, abundant in the glycans of rhEPO. They suggested that such sugar profiling may be useful in distinguishing between andogenous and rhEPO for anti-doping purposes, as well as for other medical applications.

Another immunoaffinity method to purify shEPO was developed by Lasne et al. in 2007 [78]. A column was prepared by immobilizing a

monoclonal anti-human EPO antibody (clone 9C21D11 from R&D Systems) to Affi-Gel Hz hydrazide gel from BioRad. The starting material was 4 mL of plasma collected from healthy subjects. The isolated proteins were subjected to IEF method as described for the urine samples. IEF shown that the isoelectric patterns of shEPO appeared to be highly heterogenous, being composed of more than 10 isoforms in a mean pI range of 4.1 - 4.9, slightly more basic than the pI range of 3.8 – 4.7 described for uhEPO.

2. Objectives

The rationale and motivation of the present research work arises from the difficulty to detect the abuse of rhEPOs and analogues in sport. Although two methods are available and approved by the world anti-doping agency, the IEF and SDS method. However, both are labor intensive, expensive, and lack the necessary specificity making them unsuitable for the analysis of all doping control samples generated in and out of competition.

The main pivotal objective of the present project was the development of new tools to detect recombinant erythropoietins and analogues in biological fluids, as blood or urine, based on immunorecognition.

This general objective was divided into the following specific objectives:

1. Development of a plasmatic erythropoietin immunopurification method.
2. Development of a rapid screening method for rhEPOs and analogues based on immunorecognition.
3. Development of a chip LC/MS/MS method for detection of N-glycolyl-neuraminic acid (Neu5Gc).

This research projects was performed within the framework of the following projects funded by the World Anti-Doping Agency (WADA):

- "Rapid screening (and confirmatory) method for rhEPO and NESP based on immunorecognition of its exogenous N-glycolylneuraminic acid content".
- "Detection of the non-human *N*-glycolyl-neuraminic acid (Neu5Gc) using immunopurifiation and chipLC/MS/MS.

3. Results

3.1 Purification of erythropoietin from human plasma samples as a tool for anti-doping methods

Mallorquí J, Llop E, de Bolòs C, Gutiérrez-Gallego R, Segura J, Pascual JA. Purification of erythropoietin from human plasma samples using an immunoaffinity well plate. J Chromatogr B, 2010; 878(23): 2117-22.

3.2 Recombinant erythropoietin found in seized blood bags from sportsmen

Mallorquí J, Segura J, de Bolòs C, Gutiérrez-Gallego R, Pascual JA. Recombinant erythropoietin found in seized blood bags form sportsmen. Haematologica. 2008; 93 (2): 313-314.

3.3 New screening protocol for recombinant human erythropoietins based on differential elution after immunoaffinity purification

Mallorquí J, Gutiérrez-Gallego R, Segura J, de Bolòs C, Pascual JA. New screening protocol for recombinant human erythropoietins based on differential elution after immunoaffinity purification. J Pharm Biomed Anal. 2010; 51(1): 255-9.

*3.4. Development of a screening method for rh*EPO *and analogues based on immunorecognition of its exogenous N-glycolyl-neuraminic acid content*

3.4.1. Introduction

Since 2000 the official doping control method approved by WADA to detect rhEPO abuse is the "IEF method" [45]. The method is very labour intensive (takes almost 3 days to complete a gel analysis), it is very expensive and requires a large volume of urine (i.e. ca. 20 mL). As a consequence, despite the universal EPO prohibition, the method is not applied to all urine samples collected. Therefore, since its inception, there has been an increasing interest in the development of an alternative screening method, acceptably cheap, quick and sensitive, to selectively detect the abuse of those drugs.

The most important unambiguous difference between endogenous and recombinant human EPOs and analogues produced in CHO cells is the presence of *N*-glycolyl neuraminic acid (Neu5Gc), a non-human sialic acid [54, 55], in the recombinant products [56, 57, 79]. This sialic acid cannot be endogenously produced by humans since we lack the corresponding enzyme (CMP-Neu5Ac hydroxylase). Hence, the evidence of the presence of Neu5Gc in EPO in human urine would constitute an absolute proof of its exogenous origin [80].

Different antibodies have already been described able to recognise Neu5Gc (while not Neu5Ac) present in, for example, gangliosides [64, 65]. This clearly shows that, although sugars are not considered very immunogenic, using the appropriate material it should be possible to generate monoclonal antibodies able to pick those non-human tags present in the recombinant materials.

The objective of the present work was the development of an immunoaffinity test that will result in a fast and cheap screening method to recognise the presence of the Neu5Gc moiety in the EPO molecules in order to unambiguously determine their exogenous origin. Since no commercial antibody against Neu5Gc is able to detect such moiety in

glycoproteins with the appropriate sensitivity, the production of a sensitive monoclonal antibody was required.

3.4.2. Materials and methods

3.4.2.1. Standards and general reagents

Reference preparation of rhEPO (equimolar mixture of epoetin alpha and beta) was obtained from the European Pharmacopoeia Commission, Biological Reference Preparation (BRP) batch nº 2. Darbepoetin alpha or NESP (aranesp™) was obtained as the pharmaceutical preparation from Amgen (syringe containing 10 μg of NESP in 0.4 mL solution). Pegserpoetin alpha or CERA (mircera™) was obtained as the pharmaceutical preparation (syringe containing 300 μg in 0.3 mL solution) from Roche. Monoclonal anti-hEPO antibody (clone 9C21D11) was obtained from R&D Systems. Bovine Serum Albumin (BSA), Ovalbumin (OVA) phosphate-buffered saline (PBS), MPL + TDM adjuvant, RPMI-1640 medium, Fetal Bovine Serum (FBS), 50% polyethylene glycol (PEG 1500), HAT medium supplement (50x), alkaline phosphatase conjugated anti-mouse and anti-rabbit antibodies and 4-methyl-umbeliferyl phosphate (4-MUP) were purchased from Sigma. 96 well plate maxisorp was purchased from Nunc. Gc-Free Western Blot kit was purchased from Gc-Free Inc. Chicken IgY Imperacer® kit (12-014R) were obtained from Chimera Biotech. Polyvinylidene difluoride membrane (PVDF) was obtained from Millipore. Tween-80 and Supersignal West Femto maximum sensitivity substrate was obtained from Pierce. Temed and Ammonium Persulafate were from Bio-Rad. Acrylamide-bisacrylamide (97/3, w/w) and Soduim Dodecil Sulfate (SDS) were from Merck. 240E-1 cells were kindly provided by the Laboratory of Katherine L. Knight, Chicago, IL (USA). The specific trisaccharides, Neu5Gc-Gal-GlcNac-spacer-KLH (Neu5Gc-KLH), Neu5Gc-Gal-GlcNac-spacer-OVA

(Neu5Gc-OVA) and Neu5Ac-Gal-GlcNac-spacer-OVA (Neu5Ac-OVA), later used for the production of monoclonal antibodies, were synthesized using a chemoenzymatic method by Dr. Carlo Unverzagt at the University of Bayreuth. All other chemicals were the highest purity commercially available.

3.4.2.2. Generation of MAbs

Five female Balb/c mice, of 4 - 6 weeks, were injected intraperitoneally with 40 μg of Neu5Gc-KLH in MPL + TDM adjuvant every two weeks until four immunizations. Prior and after first and third immunization, serum samples were tested for the presence of specific anti-Neu5Gc antibodies by ELISA test. Five days after the last boost, the animals were sacrified and the spleen cells were dissociated and fused with the murine myeloma cell line Sp-2.

The mouse myeloma cell line Sp-2 was grown in an enriched RPMI-10% FBS. Sp-2 cells were seeded in a T-175 flask at 10^5 cells/mL, and supplemented every two days until they reached the log-phase growth as the efficiency of the fusion has been reported to be optimal under these culture conditions.

Fusions were performed using standard methodology [81]. Briefly, lymphocytes from four of the five immunized animals and myeloma Sp-2 cells were fused at a ratio ranging from 5:1 to 2:1 in 1 ml 50% polyethylene glycol (PEG 1500) at 37ºC in serum-free medium. After several washes, cells were plated in 96 well plates at 10^5 lymphocytes per well in 200 μL RPMI + 10% FBS + HAT medium. Medium was changed every week (50% of the medium was removed and replaced with fresh medium containing HAT). After two weeks, the supernatants of wells with hybridomas were tested for the presence of specific antibodies by ELISA test.

In order to increase the odds to produce appropriate clones, the fifth immunized mouse was sent to an external company specialized in producing monoclonal antibodies (Abyntek biopharma).

Also, two female NZW rabbits were injected subcutaneously with 1,000 μg of Neu5Gc-KLH in MPL + TDM adjuvant for the primary immunization. Then, rabbits were injected every two weeks (500 μg/ rabbit) until three immunizations. Prior and after the first and third immunizations, serum samples were tested for the presence of specific anti-Neu5Gc antibodies by ELISA test. Five days after the last boost, the animals were sacrified and the spleen cells were dissociated. Lymphocytes were frozen at -80ºC for future use. Also, 100 mL of serum were obtained from each rabbit and kept at – 20 ºC.

The rabbit plasmacytoma cell line 240 E-1 were seeded in a T-175 flask at 10^5 cells/mL with enriched RPMI – 15% FBS medium, and supplemented every two days until they reached the log-phase growth.

The rabbit hybridoma production could not be done because, as some authors have described, the rabbit myeloma cell line 240 E-1 resulted unstable. Lymphocytes are frozen waiting for the availability of a new stable myeloma cell line for rabbits.

3.4.2.3. Antibody titration by enzyme-linked immunosorbent assays (ELISA)

The same trisaccharide used for immunization but linked to OVA (Neu5Gc-OVA) to avoid cross-reactions and non-specific interactions was used to coat the ELISA well plate. To be able to identify antibodies recognizing specifically Neu5Gc two ELISA tests were developed, one using Neu5Gc-OVA and another using Neu5Ac-OVA.

The compound (1 μg/well) in PBS was incubated in 96-well Nunc maxisorp for 1 hour at 37ºC. After washing with PBS, plates were blocked with 1% BSA in PBS. Serum samples or hybridoma supernatants were incubated for 1 hour at 37ºC. After washing with PBS-Tween, the secondary antibody, consisting of alkaline phosphatase conjugated anti-mouse or anti-rabbit antibodies diluted 1:1000, were added and incubated for 1 hour at 37ºC. Plates were then washed again and the substrate solution of 1 mg/mL of 4-methylumbeliferyl phosphate (4-MUP) in triethanolamine 1M buffer, pH 9.5 was added. After 30 min. the absorbance was measured at 360 nm (ref. 460 nm).

3.4.2.4. Analysis of Neu5Gc content in rhEPOs by means of a comercial antibody.

a) Western Blot

Solutions of 1 μg and 0.5 μg of rhEPO were separated using SDS-PAGE electrophoresis. Then proteins were transferred by electroblotting (0.8 mA/cm^2 gel, 30 min.) onto a PVDF membrane in a semidry blotting apparatus using a basic transfer buffer (25 mM Tris - 192 mM glycine). Then, Neu5Gc present in rhEPO was detected using the Gc-Free Western Blot kit (Gc-Free) and following the manufacturer indications. Then peroxidase substrate was added and the chemiluminescence light detected using a FUJIFILM CCD camera LAS-1000.

b) Indirect ELISA - PCR

96 Well plates from the Chicken IgY Imperacer® kit (Imperacer® kit) were used as a solid support for the immobilization of rhEPO. A 10 μg/mL solution of rhEPO in coating buffer of the Imperacer® kit (30 μL/well) was applied and incubated overnight at 4ºC. Next, wells were washed, and then blocked with blocking solution from Gc-Free (240

μL/well) overnight at 4ºC with shaking. Next day, wells were washed four times with shacking. The rest of the procedure is the same as for the sandwich ELISA protocol and is descrived below.

c) Sandwich ELISA - PCR

96 Well microtiter plates from Imperacer® kit were coated with 10 μg/mL of the monoclonal anti-hEPO antibody in coating solution. Next, wells were washed and blocked using the procedure described above. Then, 30 μL of a negative control sample (PBS) or standard samples (rhEPO, NESP, CERA and uhEPO) at 0.5 fmols/μL were added to each well and incubated during 45 min. with shaking. After removing the volume of samples, wells were washed four times with shaking.

Both ELISA-PCRs (direct and sandwich) were developed as follows:
Wells were incubated with 30 μL of IgY anti-Neu5Gc antibody from Gc-Free at 1:1000 dilution for 45 min. with shaking and then, washed four times with shaking. Next, wells were incubated with 30 μL of anti-chicken antibody-DNA conjugate from Imperacer® Kit at 1:100 dilution during 45 min. with shaking and subsequently washed six times with shaking. Wells were heated at 95ºC during 5 min. in a thermocycler to separate the DNA from the antibody. Then, supernatants were transferred to a PCR plate and 30 μL of PCR mastermix from Imperacer® kit were added to each well. Finally, PCR plates were analyzed under the following conditions:

Time	**Temp**	**Repeats**
5 min	95ºC	1
30 sec	72ºC	40
12 sec	95ºC	
30 sec	50ºC	

3.4.3. Results

3.4.3.1 Production of a monoclonal antibody specifically raised for the recognition of Neu5Gc in erythropoietin.

To get an antibody that recognize specifically Neu5Gc in rhEPO is not easy because sugars are not especially immunogenic. For this reason, mice and rabbits were immunized to obtain the required antibody.

To analyze the levels of specific anti-Neu5Gc antibodies, pre- and post-immunization serum samples from mice and rabbits were tested by ELISA assay using immobilized Neu5Gc-OVA or Neu5Ac-OVA at 10 μg/mL. Figure 1 shows that pre-immunization serum samples from all mice did not contain antibodies against the Neu5Gc-OVA. However, all post-immunization serum samples (checking one, checking two and final serum samples) strongly reacted with Neu5Gc-OVA, showing a higher reactivity after three immunizations (checking two).

The same results were obtained when serum samples were tested by ELISA assay using immobilized Neu5Ac-OVA (data not shown), indicating that the majority of the antibodies recognize equally Neu5Gc-OVA and Neu5Ac-OVA.

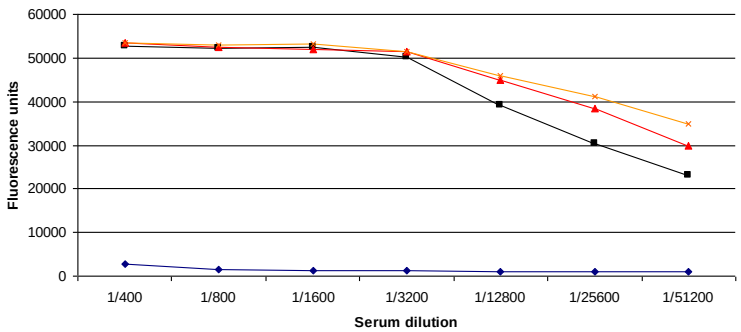

Figure 1. Antibody titters against Neu5Gc in pre-immune (blue), checking one (black), checking two (red) and final (orange) serum samples from one of the immunized mice. The same behaviour was obtained for all immunized mice.

To be sure those mice serum samples recognize our synthesized antigen and not OVA, an ELISA test was done with pre- and post-immunization serum samples using immobilized OVA at 10 μg/mL. Figure 2 shows that samples diluted 1/400 recognized OVA but in less intensity (lower of 10%) than for Neu5Gc-OVA. Also, when samples were diluted 1/1600 did not detect OVA while Neu5Gc-OVA were recognized as well as samples diluted 1/400. All these results confirmed that serum contains antibodies againts the synthesized antigen. No differences in the antibody titter and the specificity were obtained between mice immunized with the same antigen–conjugate.

Figure 3 and 4 displays results obtained for rabbit immunization assays. Rabbit post-immune serum samples strongly reacted with Neu5Gc-OVA and Neu5Ac-OVA (Figure 3), indicating a higher reactivity for both trissaccharides after the first immunization (checking one) compared to the pre-immune serum samples. The last one recognized Neu5Gc-OVA only when were diluted 1/100 and with less intensity (lower of 10%) than post-immune samples. Results showed that Rabbit-1 had more reaction against antigen than Rabbit 2, data not shown.

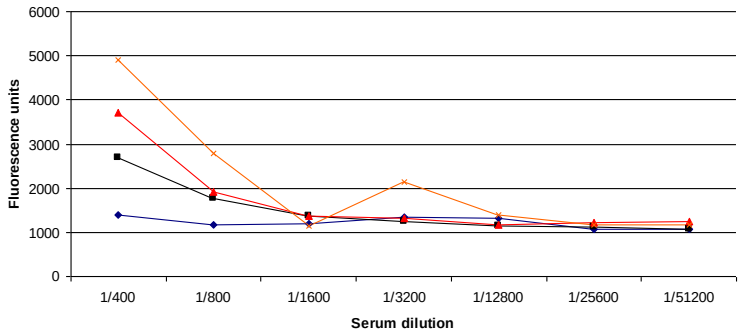

Figure 2. Antibody titters against OVA in pre-immune (blue), checking one (black), checking two (red) and final (orange) serum samples from one of the immunized mice. The same behaviour was obtained for all immunized mice.

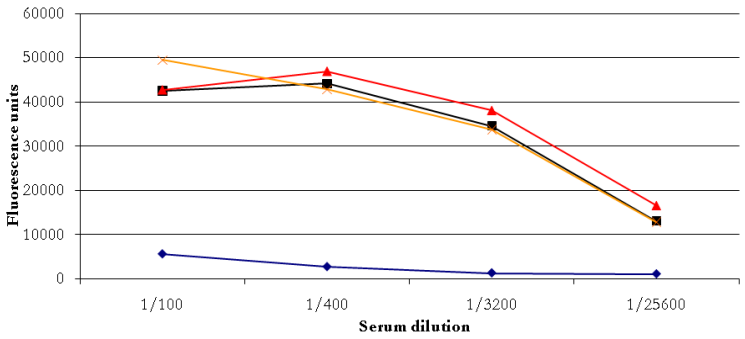

Figure 3. Antibody titters against Neu5Gc in pre-immune (blue), checking one (black), checking two (red) and final (orange) serum samples from one of the immunized rabbits. The same behaviour was obtained for the other rabbit but with less reaction against antigen.

Neither rabbit serum nor negative control (PBS) samples reacted with OVA, indicating that serum samples did not contain antibodies against OVA (Figure 4).

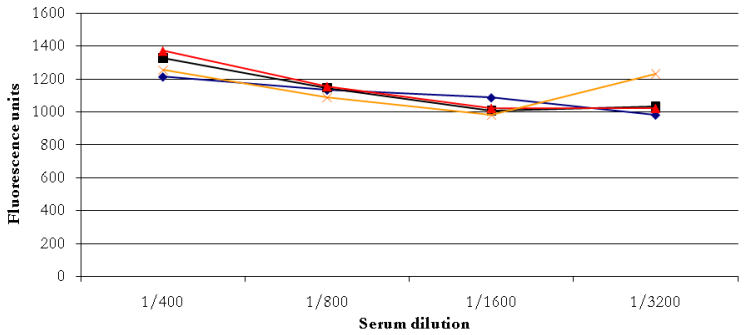

Figure 4. Antibody titters against OVA in pre-immune (blue), checking one (black), checking two (red) and final (orange) serum samples from one of the immunized rabbits. The same behaviour for the other immunized rabbit was observed.

Serum samples from mice and/or rabbits could contain a specific antibody against Neu5Gc. For this reason and considering that after three immunizations (checking two) animals had the expected titration values (pre-immune serum samples titration less than 10% of the post-immune serums titration when were diluted 1/3200), animals were sacrificed, lymphocytes obtained and, in case of mice, fusions perfomed. Rabbit fusions between rabbit lymphocytes and myeloma cell line 240 E-1 were not perfomed because the latest were not stable.

Four fusions between mouse lymphocytes and myeloma cell line Sp-2 were carried out in our laboratory and one fusion was done at the premises of the company Abyntek biopharma (mouse 2D). Figures 5, 6 and 7 show the evolution of cells during the first two weeks after fusion.

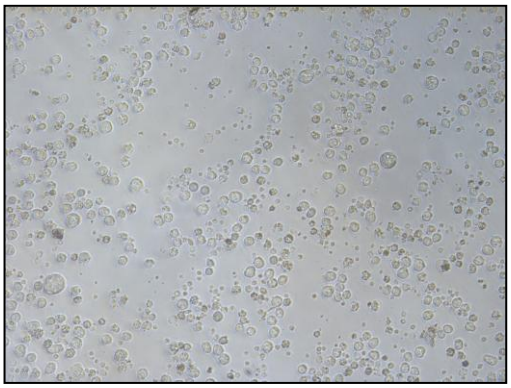

Figure 5. First day after fusion.
All cells were alive because were harvest with free HAT medium, so lymphocytes, myeloma cells and fused cells could survive at these conditions.

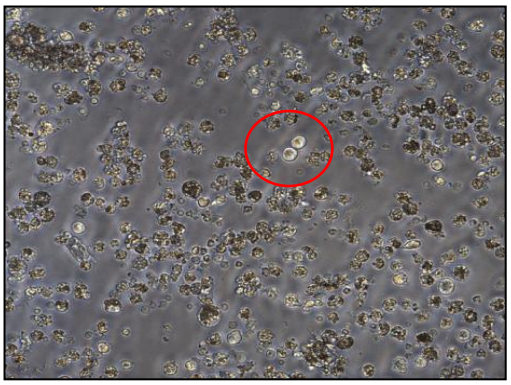

Figure 6. Mice hybridomas, four days after fusion
All cells were dead except fused cells (encircle) because were resistant to HAT enriched medium.

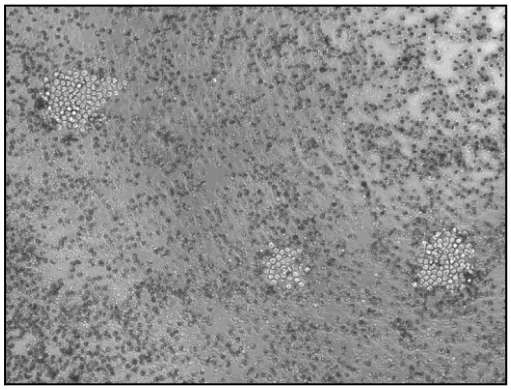

Figure 7. Mice hybridomas, two weeks after fusion. The fused cells growed.

From five different fusions, a total of 89 hybridomas were obtained (hybridomas) and supernatants were screened against Neu5Gc-OVA and Neu5Ac-OVA by ELISA test. 62 of them produced antibodies recognizing both trissaccharides (positive hybridomas) and only 4 hybridomas produced antibodies capable of reacting specifically with Neu5Gc-OVA (specific hybridomas) (Table 3).

Table 3. Number of total hybridomas (hybridomas), hybridomas that produce antibodies recognizing Neu5Gc-OVA and Neu5Ac-OVA (positive hybridomas) and hybridoma that produce specific antibodies for Neu5Gc-OVA (specific hybridomas) obtained for each mice. *Fusion perfomed in Abyntek biopharma.

Mouse	Hybridomas	Positive hybridomas	Specific hybridomas
1D	20	20	0
1E	23	23	0
1D1E	13	0	0
N	0	0	0
2D *	33	19	**4**

Figure 8 displays the difference between ELISA values obtained for the antibodies trittation against both trissaccharides (fluorescence units obtained in the ELISA for Neu5Gc-OVA – fluoresecene units obtained

int the ELISA for Neu5Ac-OVA) from the 19 positive hybridomas of mice 2D. From them, clones 72E4, 73G8, 76H9, 77A2 and 78G1 (marked with *) had much higher antibody titration for Neu5Gc-OVA than for Neu5Ac-OVA (less than 10% of the positive control signal). We consider that these hybridomas produce antibodies specifics for Neu5Gc.

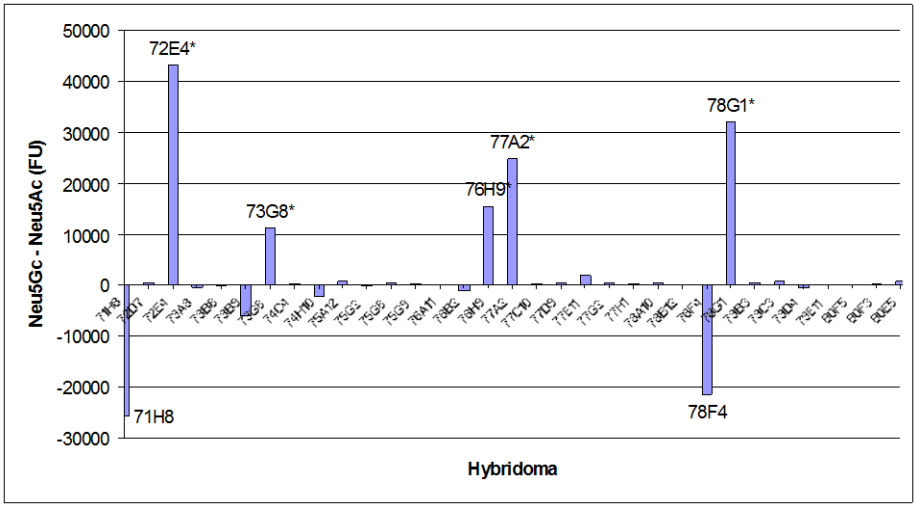

Figure 8. Antibodies specificity represented as the difference between Neu5Gc and Neu5Ac recognition values for all hybridomas of mouse 2D. (FU = fluorescence units).

After 7 - 10 days of fusion, the five most promising hybridomas were transferred from a 96 - well plate to a 24 - well plate with the aim to be cloned by the limiting dilution method. Unfortunately, after this step two of the clones did not grow and the other three stopped producing antibodies recognizing Neu5Gc-OVA.

3.4.3.2. Detection of Neu5Gc in rhEPO

A western blot (WB) kit containing a polyclonal IgY antibody specifically raised against Neu5Gc was launched by Gc-Free, Inc. The sensitivity of this kit for the detection of this non-human sialic acid in rhEPO was tested in house. Results depicted in figure 9 shows that anti-Neu5Gc

antibody could detect Neu5Gc content in 1 μg of rhEPO (average 0.1 mols of Neu5Gc per mol of proteins). However, 0.5 μg of rhEPO could not be recognized, indicating that LOD of this antibody for rhEPO was around 3.3 pmol of Neu5Gc, comparable to LOD claimed by the kit manufacturer, 5 pmol of Neu5Gc[82].

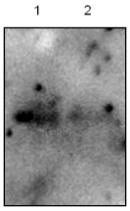

Figure 9. Detection of Neu5Gc present in rhEPO using the commercial western blot kit containing a polyclonal anti-Neu5Gc antibody. (1) 1 μg of rhEPO, (2) 0.5 μg of rhEPO.

Given the lack of sensitivity of the commercial anti-Neu5Gc antibody by western blot and attending to the need for an extreme sensitivity to detect the minute amounts of Neu5Gc expected in rhEPO, a more sensitive technique consisting in an Immuno-PCR was also tested [83].

Firstly, an indirect ELISA-PCR was developed. In this apporach, rhEPO was directly immobilized to the ELISA plate with the aim to detect Neu5Gc in rhEPO using the IgY anti-Neu5Gc antibody combined with the Chicken IgY Imperacer® kit. PCR amplification plot depicted in figure 10 shows that DNA was amplified after 22 amplification cycles, indicating that Neu5Gc contained in 0.3 μg of rhEPO was detected. It has to be pointed out that using this technique; negative controls (in the absence of Neu5Gc) should be detectable after cycle 32 [84].

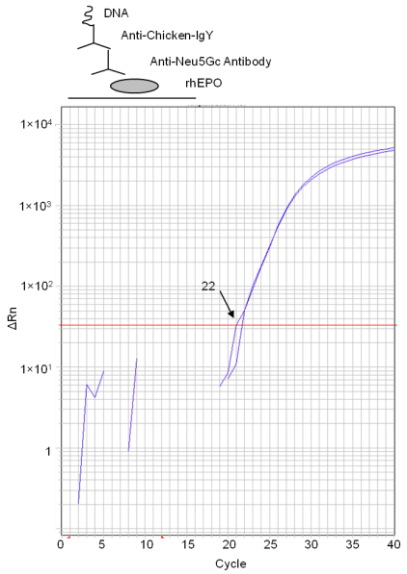

Figure 10. Amplification plot (normalized fluorescence reporter signal, ΔRn, for each PCR cycle using the direct ELISA-PCR protocol (0.3 μg of rhEPO immobilized in one well)).

For the real use of the test, a sandwich ELISA-PCR to capture the EPO contained in a biological sample is required. In this case, the monoclonal anti-hEPO antibody clone 9C21D11 was used as a capture antibody and the combination of IgY anti-Neu5Gc antibody with the Chicken IgY Imperacer® kit were used to detect the Neu5Gc present in rhEPO and analogues.

The analysis of different recombinant EPOs and analogues by the sandwich ELISA-PCR resulted in the amplification plots showed in figure 11. The negative control (left amplification plot) was amplified after 17 or 18 cycles, exactly as the samples containing rhEPO or analogues (right amplification plot). It must be concluded that there was unspecific binding masking the reaction. Either the blocking agent or the anti-hEPO antibody may contain Neu5Gc recognized by the test.

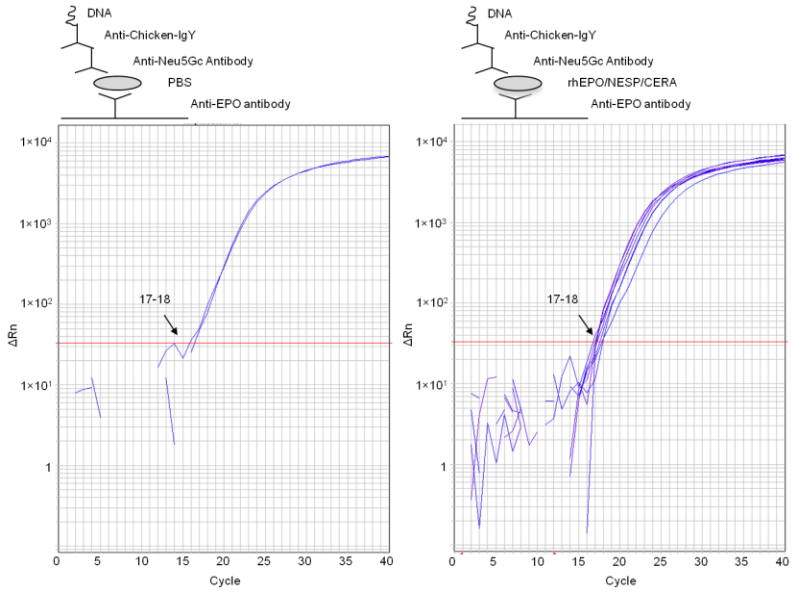

Figure 11. Amplification plot (normalized fluorescence reporter signal, ΔRn) for each PCR cycle of a "negative" sample (PBS) (left plot) and standard samples as 150 fmol rhEPO, NESP, CERA and uhEPO (right plot) analyzed using the sandwich ELISA-PCR protocol.

In order to evaluate and eliminate the unspecific binding, two experiments were performed:

- In the first, negative sample (PBS) was analyzed by the sandwich ELISA-PCR where wells were directly treated with the blocking reagent (without anti-hEPO antibody) (Figure 12, left plot)
- In the second one, negative sample (PBS) was analyzed by the sandwich ELISA-PCR where the anti-hEPO antibody (clone 9C21D11) was de-sialylated (incubation in 3 M acetic acid for 1, 2, 3 and 4 h.) prior to the immobilization to the plates. (Figure 12, right plot)

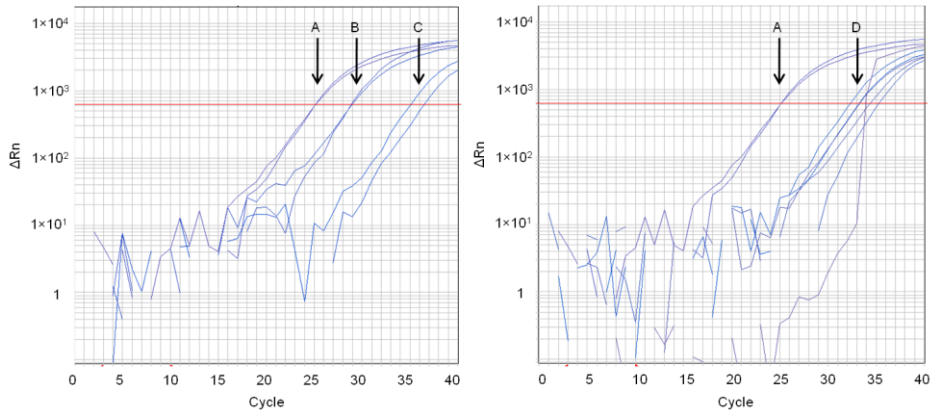

Figure 12. Amplification plot (normalized fluorescence reporter signal, ΔRn) for each PCR cycle. *Left plot*: (a) negative sample (PBS) analyzed by sandwich ELISA protocol, (b) rhEPO detected by direct ELISA protocol and (c) negative samples (PBS) analyzed using the sandwich ELISA protocol but without capture antibody, incubating the blocking reagent first. *Right plot*: (a) negative sample (PBS) analyzed by sandwich ELISA protocol, (d) negative sample (PBS) analyzed by the sandwich ELISA protocol where the capture antibody was de-sialylated using acetic acid 3 M for 1, 2, 3 and 4 hours.

When a negative samples was analyzed by the sandwich ELISA protocol directly blocked (Figure 12, left plot, curve "c"), the DNA amplification started only after 35 cycles meaning that Neu5Gc was absent. Hence, the blocking reagent cannot be the cause for the unspecific binding. When the anti-EPO antibody was immobilized tho the plate, the Neu5Gc content resulted to be higher than when it was rhEPO (Figure 12, left plot, curves a and b, respectively). This result could be explained because the capture antibody was from mice and these animals produce Neu5Gc endogenously [85]. The latter hypothesis was confirmed when a negative sample (PBS) was analyzed by the sandwich ELISA protocol where the capture antibody was de-sialylated before use (Figure 12, right plot, curve "d"). The DNA amplification was shifted to cycle 32 to 35 meaning that no Neu5Gc was detected. However, when rhEPO was analysed using de sandwich ELISA-PCR protocol with the de-sialylated capture antibody,

DNA amplification took place also at cycle 35. This result may mean that the antibody is unable to recognize rhEPO after the de-sialylation treatment.

3.5. Detection N-glycolyl-neuraminic acid by HPLC-Chip /MS/MS

3.5.1. Introduction

Sialic acids are a family of nine-carbon carboxylated sugars, distributed in the mammalian glycoconjugates such as glycoproteins and glycolipids. Sialic acids present an extreme diversity [86] and diverse roles such as the regulation of the immune response, the progression and spread of human malignancies and the microbe binding that lead to infections [87, 88, 89].

Sialic acids have an impact on half-life of pharmaceuticals products [16], thus their analysis is relevant for glycosilation quality control monitoring in marketed protein drugs [90]. Recombinant glycoproteins expressed in non-human cells and in particular rhEPOs and analogues like NESP have shown to contain small amounts of *N*-glycolyl-neuraminic acid (Neu5Gc) [56, 80], a sialic acid for which humans are devoid of the suitable hydroxylase. Presence of Neu5Gc is likely widespread in many biopharmaceutical products and could potentially play a part in immune responses against such agents [62].

This scenario of quality control of therapeutics together with the possibility of detecting the abuse of some recombinant glycoproteins by athletes [81] demands high resolution separation techniques for sialic acids and high sensitivity. Our group has already confirmed the presence of such monosaccharide in rhEPO alpha and beta as well as NESP [80, 91]. We later developed a capillary HPLC method with fluorescence detection for the determination of small amounts of Neu5Gc (LOD ca. 6 fmol) [91]. The detection of such non-human component in EPO (or other proteins) constitute an unequivocal evidence of their exogenous origin. Furthermore, according to anti-doping regulations, the use of mass spectrometry is prefered, or required whenever possible [92].

In this chapter, a highly sensitive HPLC-Chip/MS/MS method, for the determination of different sialic acids in pharmaceutical products, known to be used in sport, is described.

3.5.2. Materials and methods

3.5.2.1. Standards and chemicals

Reference preparation of rhEPO (equimolar mixture of epoetin alpha and beta) was obtained from the European Pharmacopoeia Commission, Biological Reference Preparation (BRP) batch nº 2. Darbepoetin alpha or NESP (aranesp™) was obtained as the pharmaceutical preparation from Amgen (syringe containing 10 μg of NESP in 0.4 mL solution). Pegserpoetin alpha or CERA (mircera™) was obtained as the pharmaceutical preparation (syringe containing 300 μg in 0.3 mL solution) from Roche. Epoetin delta (dynepo™) was obtained as the pharmaceutical preparation from Shire Pharmaceuticals. 1,2-diamino-4,5-methylenedioxy-benzene (DMB), 2-mercaptoethanol and *N*-Acetyl-D-neuraminic acid-1,2,3-$^{13}C_3$ ([$^{13}C_3$]Neu5Ac) were from Sigma. *N*-glycolyl-neuraminic acid (Neu5Gc) and *N*-acetyl-neuraminic acid (Neu5Ac) were from Calbiochem. All other chemicals were of the highest purity commercially available

3.5.2.2. HPLC-Chip/MS/MS system

DMB-derivatives of sialic acids were analysed on an Agilent 1200 nano-chipLC consisting of a nanoflow pump, autosampler, an auxiliary capillary pump, and an HPLC-Chip Cube interface coupled to a 6410A triple quadrupole mass spectrometer. The LCchip from Agilent contained a built-in 7.1 mm (40 nL) trap column and a 43 mm x 75 μm ID separation column, both packed with a 80 Å, 5 μm Zorbax SB C18 material, together with the nanospray needle tip. A 5 μl sample aliquot (always kept at 4ºC) was loaded onto the trap column using the capillary pump at 4 μL/min of 0.1% aqueous formic acid (FA) for two minutes. Elution from the trap column and online separation in the analytical column took place using the nanopump at 0.5 μL/min under gradient conditions rising from 100

% solvent A (0.1% aqueous formic acid) to 90% solvent B (0.1% formic acid in acetonitrile) in 1 min and held for 4 min. After returning to initial conditions, the column was reconditioned for 2 min under initial conditions before next analysis. The overall runtime was of 9 min.

DMB-Neu5Gc, DMB-Neu5Ac and DMB-[$^{13}C_3$]Neu5Ac were determined using multiple reaction monitoring (MRM) in positive mode, with N_2 as drying gas at 300 ºC and 4 L/min. The analytical conditions were set to 1800 V spray voltage and 20V collision energy. The MRM transitions finally selected were m/z 442 → 313, m/z 426→313 and m/z 429→316 respectively. Data acquisition and processing was done using the Qualitative Agilent MassHunter Workstation software.

3.5.2.3. HPLC/MS/MS system

DMB-sialic acids were also analysed using a conventional HPLC/MS/MS with an Agilent 1200 HPLC system and 6410A triple quadrupole mass spectrometer coupled through an electrospray (ESI) interphase. An analytical column acquity uplc HSS T3 C18, 2.1 x 50 mm, 1.8 μm from Waters was used. A 10 μl sample aliquot (always kept at 4ºC) was injected at 0.4 mL/min under gradient conditions starting with 100% solvent A (0.1% aqueous FA) and rising up to 20% solvent B (0.1% FA in acetonitrile) in 2 min, and kept for 2 min. After returning to initial conditions, the column was reconditioned for 3 min under initial conditions before next analysis. The overall runtime was of 9 min.

DMB-Neu5Gc, DMB-Neu5Ac, DMB-[$^{13}C_3$]Neu5Ac and were determined using multiple reaction monitoring (MRM) in positive mode, with N_2 as drying gas at 300 ºC and 6 L/min and nebulizer at 15 psi. The analytical conditions were set to 4000 V spray voltage and 20V collision

energy. The same analytes and transtions used for the ChipLC system were used.

3.5.2.4. Calibration and quality control solutions

Standard stock solutions of Neu5Ac, Neu5Gc and [$^{13}C_3$]Neu5Ac were prepared in water at 100, 100 and 1 pmols/μL respectively. Solutions were aliquoted and stored at -20ºC until used.

Calibrations curves containing Neu5Gc and Neu5Ac in a 1:100 proportion (similar to what is expected in recombinant EPO preparations) were prepared with the following Neu5Gc amounts: blank, 0.1, 0.2, 0.4, 0.8 and 1 pmol. Quality control samples (QC samples) were prepared at two different concentrations, low control (LQC) at 0.15 pmol (plus 15 pmol Neu5Ac) and high control (HQC) at 0.9 pmol Neu5Gc (plus 90 pmol Neu5Ac). All samples additionally contained 1 pmol [$^{13}C_3$]Neu5Ac used as internal standard (IS).

3.5.2.5. Pharmaceutical products

Aqueous solutions of the pharmaceutical products of of rhEPO, NESP, CERA and Dynepo were prepared at 0.6 pmols.

3.5.2.6. Sample preparation

Sialic acids need to be released from the carbohydrate chains and derivatised with DMB prior to their analysis [93]. Briefly, 3 μL of each sample were hydrolysed by addition of 2 μL of trifluoroacetic acid (TFA) 0.25 M and incubation at 80ºC for 1 hour. After hydrolysis, 5 μL of 7 mM DMB solution in 5 mM aqueous trifluoroacetic acid containing 18 mM sodium hydrosulphite and 0.75 M β-mercaptoethanol were added, mixed, vials capped and incubated at 50ºC for 2 hours with occasional mixing. Samples were stored at 4ºC until analysis.

3.5.2.7. Method validation

Calibration curves for each analyte (Neu5Gc or Neu5Ac) were obtained by least-squares linear regression analysis of the peak area ratios (area analyte/area internal standard) plotted against the analyte (Neu5Gc or Neu5Ac) amount.

The limit of detection (LOD) was calculated by analysis of spiked blank samples at different concentrations near the expected LOD and determining the minimum concentration at which analyte could be reliably detected.

Precision and accuracy were evaluated at the two concentrations of the QC samples (LQC and HQC). Precision was expressed as the relative standard deviation (RSD) of the concentration values obtained. Accuracy was expressed as the percentual difference between the calculatd and expected concentration.

3.5.3. Results

The aim of the present work was the development of an LC/MS method for the detection and quantification of Neu5Gc to be used for the testing of pharmaceutical products and, if possible, for biological samples obtained after the administration of those products. An HPLC-Chip approach was assayed, as compared to the conventional HPLC/MS/MS, in order to maximize sensitivity as this sialic acid is present in very low concentrations.

3.5.3.1. HPLC-Chip/MS/MS *vs* HPLC/MS/MS

Under the conditions used, chromatographic resolution between DMB-Neu5Gc and DMB-Neu5Ac was better for the HPLC method. An equimolar mixture of those substances was analysed using both methods (100 pmol each in scan mode). The chromatograms obtained when

monitoring both $[M+H]^+$ ions, at m/z 442 for DMB-Neu5Gc and 426 for DMB-Neu5Ac, are shown in Figure 1. Using the longer uplc column, almost baseline resolution was achieved, but the with the ChipLC column used it was not possible to separate them. Interestingly, the response of both substances (area of their chromatographic peaks) was similar under the HPLC/MS/MS conditions while using the HPLC-Chip/MS/MS approach DMB-Neu5Gc response was much weaker. However, the overall sensitivity was always better using latter.

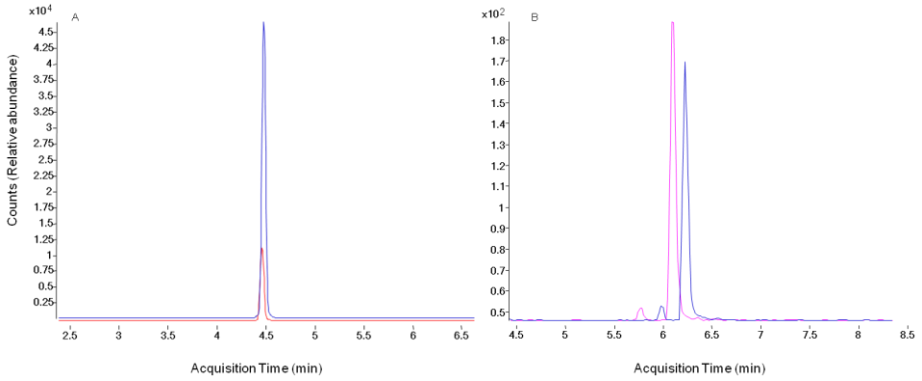

Figure 1. Extracted ion chromatograms at m/z 442 and m/z 426 obtained for the analysis of DMB-Neu5Gc (red trace) and DMB-Neu5Ac (blue trace), scan m/z 200-450 in the positive mode), using HPLC-Chip/MS/MS (A) and HPLC/MS/MS (B).

Because of the potential impact of the expected difference in concentration of both substances in real samples or pharmaceutical products, were also performed of mixtures Neu5Gc: Neu5Ac in a 1:100 molar ratio. Results are shown Figure 2 where 1 and 100 pmol of the respective compounds were injected. The signal to noise ratio for Neu5Gc using the HPLC-Chip/MS/MS and HPLC/MS/MS methods was 371.5 and 22.9 respectively. For this reason, the HPLC-Chip/MS/MS method was used in all experiments.

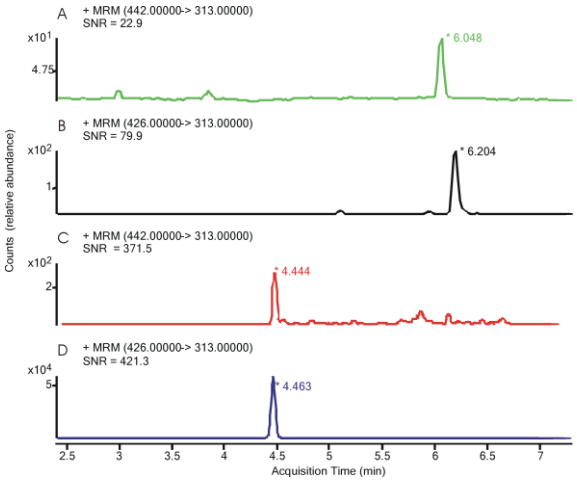

Figure 2. Extracted ion chromatograms from MRM analysis of DMB-Neu5Gc and DMB-Neu5Ac at 1 pmol and 100 pmols on column, respectively, using HPLC/MS/MS (A and B) and HPLC-Chip/MS/MS (C and D) methods. A) Neu5Gc (precursor ion m/z 442, product ion m/z 313); B) Neu5Ac (precursor ion m/z 426, product ion m/z 313); C) Neu5Gc (precursor ion m/z 442, product ion m/z 313); D) Neu5Ac (precursor ion m/z 426, product ion m/z 313)

3.5.3.2. Mass spectrometric identification of Neu5Gc and Neu5Ac by HPLC-Chip/MS/MS

The structure of the DMB derivatives of the analytes was confirmed obtaining the product ion scan of the $[M+H]^+$ ions at m/z 442 and m/z 426 for DMB-Neu5Gc and DMB-Neu5Ac respectively. Different collision energies 0, 10, 20 and 30 V were used (Figure 3). At collision energy 0 the loss of water $[M+H-H_2O]^+$ was already found as the only product ion for both substances (m/z 424 and m/z 408 respectively). At collision energy 10 V DMB-Neu5Gc continued not showing any further fragmentation while DMB-Neu5Ac already started to show some fragmentation. When the collision energy was raised to 20 V, the two major fragments appeared for both substances at m/z 313 corresponding to an extensive dehydration (-3 H_2O) and loss of the whole amino group

(either *N*-glycolyl or *N*-acetyl) and cyclisation. A further fragmentation of the new ring formed gave rise to a fragment at m/z 229 (Figure 3). A fragment at m/z 283 corresponding to the loss of formaldehyde was also found for DMB-Neu5Ac. This fragment was predominant at higher collision energies (30 V) for both substances, data not shown.

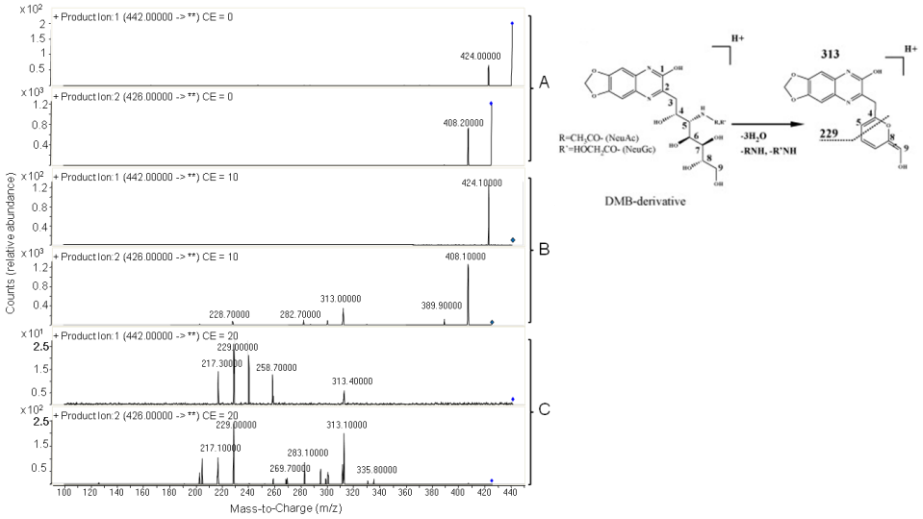

Figure 3. Product ion scan spectrum of the corresponding precursor ions at m/z 442 and m/z 426 corresponding to the $[M+H]^+$ species of DMB-Neu5Gc and DMB-Neu5Ac at different collision energies (left). A) CE = 0 V, B) CE = 10 V, C) CE = 20 V). Elucidation of the main fragments is described on the right.

From these results, the optimal collision energy was chosen as 20 V and two MRM transitions for each sialic acid were initially selected: m/z 442 → 229 and m/z 442 → 313 for DMB-Neu5Gc and m/z 426 → 229 and m/z 426 → 313 for DMB-Neu5Ac. However, it was soon observed that the sensitivity rapidly decreased with the number of transitions chosen. Furthermore, although the transition m/z 442 → 229 showed the maximum sensitivity, it resulted in a high background when real samples were analysed, thus finally the following two transitions were used: m/z

442 → 313 and m/z 426 → 313. Additionally, the analogous transition m/z 429 → 316 was used for DMB-[$^{13}C_3$]Neu5Ac used as internal standard (IS).

3.5.3.3. Validation

Calibration curves in the range 0.1 to 1 pmol Neu5Gc, containing Neu5Ac in a proportion 1:100 plus 1 pmol [$^{13}C_3$]Neu5Ac used as IS were analysed. Results are shown in Figure 4. Determination coefficients (r^2) better than 0.95 were obtained. The behaviour of the Chip was less robust than the conventional ESI interface with coefficients of variation higher than those regularly obtained in conventional set-ups.

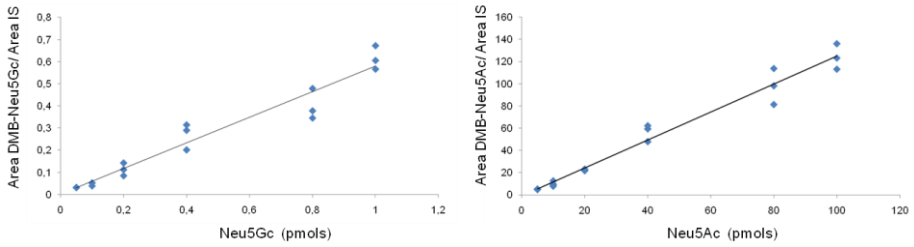

Figure 4. Calibration curves of DMB-Neu5Gc (left) and DMB-Neu5Ac (right). Amounts of both sialic acids on column (50% of the amount present in the sample).

The limit of detection for DMB-Neu5Gc was 50 fmols on column (0.1 pmol in the sample). Accuracy and precision of the method were evaluated at the two concentration levels of the quality control samples: 0.15 pmol Neu5Gc (LQC) and 0.9 pmol Neu5Gc (HQC), containing Neu5Ac in a 1:100 proportion. Relative standard deviations (RSD) ≤ 20 % for the LQC and < 15 % for the HQC were achieved inter-assay for both compounds. Accuracy, expressed as the relative difference between the nominal and calculated value was always within ± 20 %.

Figure 5 shows the extracted ion chromatogram (EIC) of a blank, LQC and HQC sample.

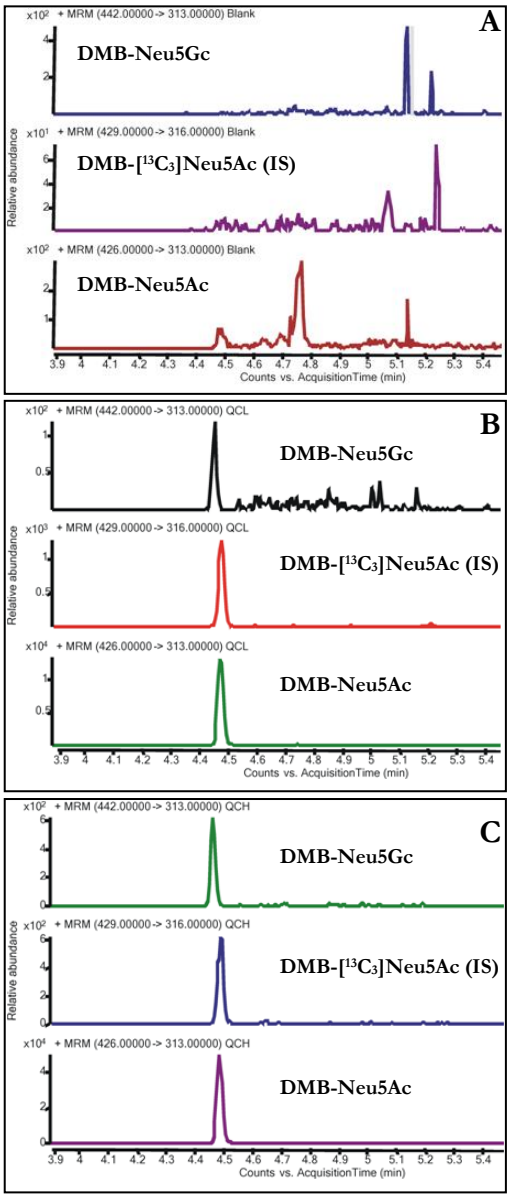

Figure 5. Extracted ion chromatograms of the anlaysis of samples containing DMB derivatives of sialic acids. A: Blank sample, B: LQC with 0.075 and 7.5 pmol of DMB-Neu5Gc and DMB-Neu5Ac on column respectively. C: HQC with 0.45 and 4.5 pmol of DMB-Neu5Gc and DMB-Neu5Ac on column respectively.

3.5.3.4. Quantification of Neu5Gc in rhEPO, NESP, Dynepo and CERA

The method was used for the analysis of pharmaceutical preparations of rhEPO, NESP, Dynepo and CERA. Sialic acids of samples containng 2 pmols of the glycoprotein were analysed in triplicate as described. The Neu5Gc characteristic transtition at m/z 442→313 clearly indicated the presence of Neu5Gc in all these pharmacological products except Dynepo. The percentage of Neu5Gc with respect to Neu5Ac found in rhEPO, NESP and CERA were 0.91 % ± 0.11, 0.89 % ± 0.16 and 1.29 % ± 0.2 respectively. As expected, Dynepo produced in human cells, did not contain Neu5Gc, at least above the limit of detection in this experiments, which would have been a 0.25%.

4. Discusion

Along the development of this work different approaches to purify and/or detect recombinant erythropoietin or analogues have been addressed. The ultimate goal was the selective detection of minute amounts of those recombinant glycoproteins in the presence of the naturally occurring endogenous EPO.

The abuse of rhEPO and analogues in sport is currently detected in urine samples by the so called IEF method [44, 45]. However, the method is affected by the protein content of the sample and additional purification steps are necessary. On the other hand, the detection of those substances in blood is sometimes imperative, because blood is the only sample available (e.g. blood bags seized by the *Guardia Civil* in the called Operation "Puerto") or because the particular compound is not readily excreted in urine, e.g. CERA [94]. Plasma or serum cannot be directly analysed by the IEF method due to its very high protein content, thus an appropriate purification step is essential. Thus, the first goal of this work was the development of an immunopurification method compatible with the requirements of rhEPO detection.

Since the 1970s all immunopurification methods developed used polymers (e.g. sepharose) or magnetic beads as solid support to attach antibodies or lectins [75-78]. Our approach was using 96 well plates as solid support because plates are disposable, easy to use and amenable to the simultaneous processing of a great number of samples avoiding cross-contamination. Additionally some are commercially available as part of EPO ELISA kits. The initial plan was starting with a commercial ELISA kit to then try to replicate or improve the results with our own custom made plates. The rationale behind the approach was that in the event that a proper antibody was developed or found against i.e. Neu5Gc a sandwithc ELISA test could have been developed on the same plate where the immunopufirication took place.

While studying the conditions to quantitatively elute the EPO retained in the wells different elution buffers and pHs were tested. It was soon realised that while trying to elute EPO, other proteins present in the commercial ELISA kits were also eluted. Quantitative measurements showed those could not be the antbodies coating the wells [95], they had to be part of the blocking reagents used. The most frequently used blocking reagents are proteins like BSA, Gelatine and in some cases glycoproteins [96]. This finding immediately suggested these contaminations would make the procedure incompatible with other analytical purposes, as sialic acid analyses. For these reason, an in-house immunoaffinity plate was developed to study the blocking, binding and elution conditions.

A non proteic blocking reagent, polyvinilpirrolidone (PVP) [97] was finally chosen. Its blocking capacity was shown to be similar to BSA and gelatine. In order to study the hEPO-antibody binding, two different incubation conditions (overnight at 4ºC and 1h at room temperature) and three different anti-hEPO antibodies (two monoclonal antibodies, clone 9C21D11 and clone AE7A5, and one polyclonal antibody (AB-286-NA), all of them from R&D systems) were studied. The final conditions chosen were the use the monoclonal antibody clone 9C21D11 and incubate overnight at 4ºC with shaking. Samples incubated for 1 hour at room temperature (conditions used in the commercial quantitation ELISA tests) resulted in a 50% decrease of rhEPO bound.

Then, attention was paid to the elution capacity of different buffers. The objective was to obtain a buffer able to disrupt the antigen-antibody binding while not degrading the analyte. Interestingly, results showed that the EPO IEF profile changed depending on the elution buffer used. The IEF profile of all rhEPOs and analogues before and after purification were the same when the elution was perfomed at a strong alkaline pH

(above 11). Conversely, when elution was performed at an acidic pH (acetic acid 0.7 %), a selective elution was achieved with an enrichment of the less acidic bands and a consequent IEF profile change with an apparent shift towards the cathode. NESP, with a very acidic profile was virtually not eluted. At first, it was considered that the acidic pH may hydrolyse the sialic acids degrading the glycoprotein, but uhEPO showed to be perfectly stable when incubated in those exact acidic conditions. Aslo this effect is not generic for any antibody as other authors could elute rhEPOs without apparent isoform discrimination under acidic conditions using different antibodies.

The experiments carried out to study the EPO recoveries suggested that working with normal plasmatic EPO concentrations, 50% of the plasmatic EPO is retained by the antibody. This retention did not discriminate between isoforms because the IEF profile of the unbound sample (fraction of EPO which did not bind to the plate) and the initial sample were identical. Also, these experiments suggested that with the acidic buffer, only 50 % of the retained EPO is eluted, so, 25 % (the most acidic bands) of the total EPO presented in a sample is recovered using this buffer. However, with a basic buffer, all EPO retained is eluted, corresponding to the 50% of the initial EPO.

After ensuring no isoform discrimination, we could see that plasma hEPO may show a profile slightly more basic than the regular uhEPO. Several reports on endogenous EPO have shown that circulating EPO contains fewer acidic glycoforms than urinary EPO, speculating that the charge difference could be attributed to a difference in renal handling of the various glycoforms or post-secretion processing of the glycans [98]. However, glycan structures responsible for those differences have never been reported.

The last important factor to be evaluated was the clean-up efficiency; Total protein content in the eluate was reduced three thousand times, while half of the EPO was recovered. However, as proteins are present in plasma in much higher amounts than EPO, one step immunopurification is not enough to obtain a highly purified EPo for glycan identification, for example. At present, only sialic acids analyses, IEF profiles and Mw determinations by western blot can be applied to biological samples. No structural characterisation can be done. The sensitivity limitations of analytical instruments and the low amounts of EPO present in biological fluids (~10 ng/L in urine and ~100 ng/L in serum) [47] are the main reasons for failing in revisiting structural investigations on endogenous EPO.

To proof that EPO from plasma processed with the developed method is useful to detect rhEPO using the IEF method, plasma samples with supra-physiological concentrations (suspicious of rhEPO abuse) from the Operation "Puerto" were analyzed using the developed method. Results showed that those samples had profiles compatible with the presence of rhEPO, while a blank plasma profile, although slightly shifted toward the cathode, was far from complying with the identification criteria for rhEPO as described in WADA's TD2009EPO [99].

Interestingly, plates used did not contain any non-human proteic material that could be eluted from the wells and its single use avoids cross-contamination between samples. So the immunopurified samples could be analysed for the presence of Neu5Gc, the non-human sialic acid present in recombinant glycoprotein preparations [56, 57]. The presence of Neu5Gc in immunopurified biological samples would unambiguously indicate the presence of exogenous erythropoietin. An HPLC-FLD analysis of the DMB derivatized sialic acid residues hydrolysed from hEPO immunopurified from control plasma samples was carried out. The

same samples were also evaluated by IEF to cross-check both results. As expected, results revealed absence of Neu5Gc in blank plasma used as a negative control while this non-human sialic acid was detected when the blank plasma sample was spiked with rhEPO. Analogously, Neu5Gc was detected in those suspicious plasma samples with supraphysiological EPO concentrations where IEF profiles already were compatible with the presence of rhEPO.

One of the major objectives of this work was addressing the issue of the lack of a proper screening method, a method sufficiently quick and sensitive to be applied to all samples collected for doping control. Those features represent the major drawback of the current IEF method.
While studying immunopurification it was immediately realized that the pH-dependent selective elution of EPO isoforms from an immunoaffinity plate could be used to readily differentiate between rhEPO and uEPO.
Under acidic conditions, a greater proportion of basic bands is eluted while under basic conditions no discrimination is produced. Consequently comparing both fractions it could be obtained a measure of the band distribution. For rhEPO with bands appearing just in the basic area of the gel, the acidic fraction will account for almost 100% [100] of the bands while for uEPO, with bands spread all through the different pH areas of the gel, it would represent a much lower proportion. The same reasoning applies to CERA or Dynepo. For NESP the situation would be the opposite with a very low recovery under acidic conditions, much lower than for uEPO. The approach followed to use this principle required two steps: an isoform selective immunopurifaction and the EPO quantification. In order to ease the use of this methodology making it independent of a particular custom made immunopurification technique, a commercial ELISA plate was chosen for the immunopurification step

[101]. For EPO quantification, and in order to add an orthogonal dimension to the approach, Immulite 1000™ was used instead of other conventional ELISA techniques. Furthermoe, Immulite provides a much faster and automated determination [102]. The whole screening method could be done in only half a day.

Results confirmed that rhEPOs and CERA (more basic profile) were predominantly eluted at acidic pH, showing a higher recovery in that fraction than samples containing only urinary EPO (profile shifted towards more acidic pI values). However, NESP (having the whole isoelectric profile in the "acidic area") were predominantly eluted at basic pH, showing a lower recovery in the acidic fraction [100]. In addition, mixtures with different proportions between rhEPO and uhEPO were also tested in order to simulate the situation encountered in real urine samples. From those experiments, it could be concluded that changes in those relative recoveries could also be observed when proportions rhEPO to uhEPO were changed.

In order to compensate for the day to day variation uhEPO reference standard was analysed in parallel in each batch and taken as a kind of internal standard [103]. The ratio between the amount of EPO eluted in the acidic and basic fraction was calculated for uhEPO in each batch. The values obtained for the unknown samples were given relative to the uhEPo value. This was called "ratio QA" being 1 for uhEPO, by definition, above 1 for all those forms of rhEPO with a higher proportion of basic isoforms and much below 1 for the hyper acidic analogue NESP.

Once those differences between recombinant and endogenous erythropoietin relative recoveries were evidenced, studying the range of population values for the chosen marker (ratio QA) that could be obtained and then derive a cut-off value from it was adressed. The mean population value found for the ratio QA was below the very uhEPO

value, 0.86 with a standard deviation of 0.15. This result suggests that there is a matrix effect for urine, not counted for when analysing uhEPO standard or even that there may be differences in behaviour of that standard, obtained from anaemic patients, and the endogenous urinary EPO found in healthy individuals. Also, this result shows that EPO isoforms abundance could be different between people [104].

From the values obtained for blank (negative) urine samples, the cut-off value for the ratio QA covering the 95% conficence interval would be 1.15 and 0.57. In our experiments samples containng a 25% of rhEPO mixed with 75% of uhEPO could be detected while this combination would not or barely comply with the identification criteria of TD2009EPO. The major drawback of the approach was the lower boundary of the population. It was set at 0.57 meaning that a result below that number should indicate the presence of NESP. It was seen that only when NESP was present in high proportions the method was able to pick it up. One of the causes of this low differentiation between endogenous EPO and NESP could be due to the very NESP quantification by IMMULITE 1000^{TM} . It was found that under the conditions used, values obtained for NESP in the basic fraction tended to be lower than expected, thus affecting the ratio. Other quantification methods should be explored, both to increase the reliability of the values found and also to increase the sensitivity, thus requiring less starting material [105].

Finally, in order to consider the potential urine matrix effect, increasing amounts of rhEPO were added to blank urine retentates and taken through the new screening procedure as well as analysed by the current IEF method. Results showed that all samples identified by the IEF method as containing rhEPO were picked up by the new screening procedure with ratio QA values above the cut-off. Considering these

results, the newly developed procedure has shown similar or slightly better sensitivity than the current IEF method for rhEPO.

Another important finding was that the unbound fraction of this immunopurificaiton procedure does not show isoform discrimination, thus it could also be analysed by the IEF method EPO.

This screening method has some drawbacks that need to be discussed. As it roughly inidicates distribution of bands, shifted profiles obtained after certain particular effort conditions ("atypical profiles") or after degradation ("active urines") would be picked as suspicious. This problem also occurs with the IEF method where cautious identification criteria have to be applied to avoid misinterpreting those unfrequent profiles [106]. On the contrary, SDS-PAGE would not significantly be affected by those effects [49] and is currently being explored as an alternative confirmation procedure. However, while CERA and NESP can be clearly differentiated due to its higher molecular weight, more efforts must be dedicated to improve the SDS resolution allowing unequivocal differentiation between rhEPOs and uhEPO.

There is another way to unambiguously discriminate between recombinant and endogenous EPO molecules. Amongst the more than 60 natural analogues of sialic acid described, *N*-acetyl-neuraminic acid (Neu5Ac) is by far the most common sialic acid species [107]. Conversely, *N*-glycolyl-neuraminic acid (Neu5Gc) cannot be produced by humans. Since rhEPOs are synthesised in CHO or BHK cells they shall contain small amounts of this non-human sialic acid [56, 57], as it occurs frequently in animal cells [58, 59]. Dynepo, produced in a human cell line would, in principle, not share this feature. Besides, it should not be excluded that the ingestion of animal products, such as red meat and milk, could also introduce trace amounts of this residue into human proteins

[108]. In principle, the finding of Neu5Gc in EPO would unambiguously indicate its exogenous origin, thus being an ideal method. Monitoring this non-human sialic acid in biopharmaceutical products could also be of great interest since it may be linked with autoimmune response episoded and chronic inflamation already described in humans [109].

So, the third objective of the present thesis was to develop a method to detect and identify Neu5Gc. Two different methods were explored to achieve the objective. The first idea was to develop an immunological method to detect the specific antigen and the second one was to follow a chemical approach and develop a highly sensitive HPLC-Chip/MS/MS method.

Regarding the immunological approach, we tried to develop a monoclonal antibody specific for Neu5Gc, i.e. selectively recognising this sialic acid while not cross-reacting with the most abundant Neu5Ac. When the project started, no commercial antibodies against Neu5Gc were available found in the market. Other groups had already described the production of monoclonal antibodies against this antigen in lipids [64, 65].

So, even though these moieties are considered not ver immunogenic, it seemed it could be done provided the appropriate immunogen is used. To that end, rabbits and mice were immunised with a trisaccharide containing Neu5Gc conjugated to KLH (Neu5Gc-KLH). All animals produce antibodies able to recognise the trisaccharide, as tested using Neu5Gc-OVA instead of the immunogen. However, antibody titrations showed that they were able to recognised both Neu5Gc-OVA and Neu5Ac-OVA. Although some hibridomes were obtained apparently producing selective antibodies, none produced antibodies specific against Neu5Gc. New attempts with the help of a specialised company (Abyntek Biopharma) resulted in the selection of four clones that produce specifically antibodies

against Neu5Gc. Unfortunately, two of the clones finally did not grow and the other three stopped producing antibodies recognizing Neu5Gc.

So it seems it could be possible to achieve this goal and new attempts should be performed as it would be an invaluable tool for the differentiation between rhEPO and endogenous EPO.

At that time, a commercial polyclonal antibody against Neu5Gc produced in chicken appeared in the market [82]. Chickens, as humans, lack the enzyme CMP-Neu5Ac hydroxylase and for this reason have a good immunogenic response towards Neu5Gc [110]. The antibody had been tested by western blot with a sensitivity of detecting 5 pmol Neu5Gc.

That is the amount expected to be present in ca. 50 pmol rhEPO (approximately 1.5 μg rhEPO or 180 IU). This result suggested that the polyclonal antibody against Neu5Gc could be useful for other purposes, like cancer diagnostics, etc. but not for our purposes. Our ultimate goal is detecting Neu5Gc in the range 1-10 fmol, the amount that could be present in a reasonable volume of urine (i.e. 20 mL) [111]. Still, trying to take profit of this unique antibody, we tried to amplify the signal by using a PCR amplification kit designed for ELISA (Imperacer™). Though different experiments were done with the aim to detect these lower amounts of Neu5Gc, no positive results were obtained. The biggest difficulty was that antibody detects also the Neu5Gc present in the capture antibodies of the ELISA wells (mouse monoclonal anti-EPO antibody, clone 9C21D11) making the approach impossible.

The chemical approach, i.e. identifying Neu5Gc by mass spectrometry was still an alternative to explore. A very sensitive method would be required and for this reason, a HPLC-Chip/MS/MS method was tested as compared to a conventional HPLC/MS/MS approach. In principle 5 fmols of an analyte would be within the sensitivity of the equipment as

other analytes are publisized as detected in the low attomol range (e.g. atropine) [112].

The method consisted of a hydrolysis to release the sialic acids from the glycoprotein followed by derivatisation with a fluorescence group, DMB. This derivatisation is specific for alpha keto acids, making it ideal for the clean-up of the sample. Furthermore, it confers btter chromatrographic behaviour to the compounds. Then DMB-Sialic acids would detected by LC/MS.

As expected, the best sensitivity was obtained when sialic acids were analysed with an HPLC-Chip/MS/MS system where a nano LC column and a built-in nanospray needle is used. A limit of detection of 50 fmols Neu5Gc injected was achieved. This sensitivity was twenty times higher than what could be obtained by a conventional HPLC/MS/MS approach with an electrospray interphase. A sensitivity increase in the order of 300 times was expected because of the change in the dimensions of the system and the efficiency of the nanospray. Only recent publications using nanoLC/Fourier Transform Ion Cyclotron MS were able to reach LODs in the range 6-9 fmol [62, 63].

The linearity of our method for Neu5Gc and Neu5Ac was sufficient for the purpose of quantifying the proportion in which both sialic acids were present in pharmaceutical products. It is know that the presence of this non-human sialic acid in these products could produce adverse effects in humans [113, 114], hence monitoring Neu5Gc could be a good tool as a quality control. Small amounts of pharmaceutical preparations of rhEPO, NESP, Dynepo and CERA were analysed and their Neu5Gc content quantified. As expected, Dynepo™, produced in human cells, did not contain any detectable Neu5Gc while rhEPO alpha and beta, as well as NESP yielded 0.91 mol % and 0.89 mol % with respect to Neu5Ac, respectively. These results confirm the previous findings of our group

using an HPLC-FLD approach [79] Also, the new generation of recombinant EPO, CERA, a pegylated epoetin beta [18], was shown to contain 1.3 mol %. It is the first time that Neu5Gc is detected in CERA and this result confirms that all rhEPOs and analogues produced in animal cells contain this compound.

Unfortunately, with that sensitivity, the method could not be applied to urine samples. Renewed efforts are necessary to lower LODs by at least an order of magnitude and make it compatible with the robustness of a routine method.

5. Conclusions

The conclusions drawn out of the present work are summarised as follows:

1. A hEPO-specific immunoaffinity procedure using microtiter plate was developed. This approach allows isolating erythropoietin from complex biological matrices (e.g. plasma), avoiding contamination with other non-human material and making them amenable to analytical methods such as IEF-PAGE or sialic acid analyses.

2. EPO elution from immunoaffinity plates showed to be potentially selective depending on the pH. Under acidic conditions (pH ~2) there was an obvious discrimination favouring the elution of more basic isoforms. Conversely, under basic conditions (pH ~11) there was no discrimination.

4. IEF analysis of immnopurified plasma EPO demonstrates the feasibility of using plasma to detect the administration of rhEPO. As reported for serum, it was shown that plasma EPO has an IEF profile less acidic than urinary EPO.

5. Sialic acid analyses confirmed that Neu5Gc could be detected in biological samples containing rhEPO (i.e. plasma positive sample) if this sample is immunopurified before analysis.

6. A screening procedure based on a new principle was developed. The method differentiates rhEPO from endogenous EPO profiting the isoform selective elution of EPO under acidic conditions.

7. The fraction of the sample not bound to the antibody during the immunopurification does not show any isoform discrimination and can be used for IEF analysis.

8. Rabbits and mice can be immunized to produce antibodies against trisaccharides containing sialic acids. Monoclonal antibodies selectively recognising Neu5Gc in the presence of Neu5Ac can be produced. Five mice hibridomas were obtained showing those features, although they were finally not viable. No hibridomas could be obtained from rabbit as the myeloma cell line 240 E-1 resulted being unstable.

9. A sensitive ChipLC-MS/MS method for Neu5Gc detection and quantification was developed with a limit of detection of 50 fmol. The method was successfully used to detect and quantitate the Neu5Gc content of different pharmaceutical EPO products (rhEPO, NESO, Dynepo and CERA).

10. While products produced in CHO cells like rhEPO, NESP and CERA showed to have around 1% of Neu5Gc, Dynepo™ produced in a human cell line showed not to contain any detectable Neu5Gc.

11. The sensitivity of the method resulted insufficient for the analysis of its content in urine samples.

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