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BIOSIMILARS — LINKING QUALITY DATA TO CLINICAL OUTCOMES

Biosimilars — correlatie tussen kwalitatieve data en klinische resultaten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 12 oktober 2016 des middags te 12.45 uur

door

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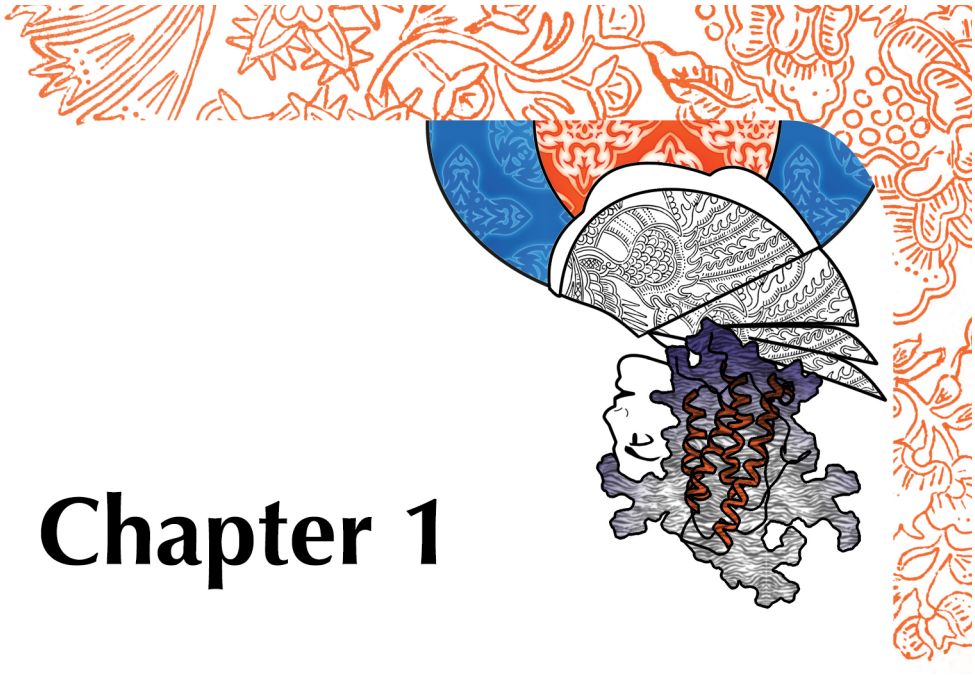

Chapter 1

General Introduction

Liem Andhyk Halim

INTRODUCTION

The use of biotherapeutics dates back to the time of the 19th century, when animal antisera were introduced to treat infections. This was followed by the use of insulin purified from bovine and porcine pancreas in 1921[1]. Due to their animal origin, these products were immunogenic, and their use sometimes led to serious anaphylactic reactions. Human-derived proteins introduced later, such as human growth hormone and human factor VIII, are also able to elicit the formation of antidrug antibodies (ADAs) caused by lack of tolerance to these proteins in patients.

Progress in recombinant DNA (rDNA) technology has made the development of (nearly) identical to native human proteins such as recombinant insulin[2] as well erythropoietin (epoetin)[3] possible. Together with hybridoma technology, these advances in science and protein manufacturing have allowed the production of monoclonal antibodies and led to the introduction of biotherapeutics to treat a variety of diseases[4].

Like all drugs, biotherapeutics eventually face patent expiration, allowing follow-on versions to be introduced. Unlike small molecules drugs, which are chemically synthesized by robust production processes and are uniform in terms of product characteristics and batch-to-batch consistency, biotherapeutic drugs are heterogeneous and complex mixtures. As a result, comparison between innovator drugs and follow-on products is extremely difficult. Furthermore, the existing analytical tools do not allow full prediction of the biological and clinical properties of biotherapeutics, including immunogenicity. Hence, the generic drug paradigm used to authorize small molecules drugs is insufficient to ensure the efficacy and safety of most biotherapeutics as initially addressed by Schellekens and Charles-Ryff[5].

In 2004, the EMA was the first regulatory agency to introduce a different regulatory framework for marketing authorization of these follow-on versions, referred to as "biosimilar"[6]. Besides extensive proof of product quality, which includes extensive comparisons of both the physiochemical and biological properties between the candidate biosimilar and the innovator product, clinical comparative PK data as well comparative clinical studies are needed. Further, the evaluation of the immunogenic potential of the biosimilar is mandatory. To date, more than 21 biosimilar products from 7 different classes have been approved in the EU based on this legislation with no reports of serious concerns.

Biosimilar epoetin and filgrastim

Hematopoietic growth factors, including recombinant human erythropoietin (epoetin) and recombinant human granulocyte colony-stimulating factor (filgrastim), were among the first rDNA-derived biotherapeutics[7].

Epoetin

Following from the cloning of the human EPO gene in 1983, epoetin alpha was marketed in Europe beginning in 1998 for the treatment of anemia in chronic kidney disease (CKD)[8,9]. Subsequently, epoetin received authorization for the treatment of anemia in cancer[10] and in zidovudine treated HIV patients[11] as well as in pre-surgery patients[12]. Other innovator epoetin products received their

marketing authorization in the EU as listed on **Table 1**. All these epoetin products differ either in glycosylation or have modifications such as hyper glycosylation or pegylation. In 2007, the EMA authorized the first two biosimilar epoetins traded, under 5 different names.

Table 1. Innovator and Biosimilar Epoetin Products on The EU Market

Brand	Active substance	Host cells	Ref.
Innovator epoetin			
Procrit® (Ortho Biologics Inc.) Eprex®/Erypo® (Janssen-Cilag Ltd)	epoetin alpha	CHO	13 14
Recormon® (Boehringer Mannheim) NeoRecormon® (F. Hoffman-La Roche Ltd.)	epoetin beta	CHODN2-3a3	15
Epomax® (Baxter)[a]	epoetin omega	BHK-21	16
Dynepo® (Shire Pharmaceuticals)[b]	epoetin delta	HT-1080	17
Biopoin® (Teva GmbH) Eporatio® (Ratiopharm GmbH)	epoetin theta	CHO-K1	18 19
Aranesp® (Amgen)	darbepoetin alpha	CHO-K1	20
Mircera® (Hoffmann-La Roche)	methoxy polyethylene glycol – epoetin beta	CHO	21
Biosimilar epoetin			
Abseamed® (Medice Arzneimittel Pütter GmbH & Co. KG) Epoetin alpha Hexal® (Hexal AG) Binocrit® (Sandoz GmbH)	epoetin alpha	CHO	22 23 24
Retacrit® (Hospira UK Ltd.) Silapo® (Stada Arzneimittel AG)	epoetin zeta	CHO	25 26

[a] Product has been abandoned
[b] Product is no longer authorized

Filgrastim

Filgrastim is an analog of the 18.8 kDa human growth colony-stimulating factor, and is recombinantly produced in *Escherichia coli* (*E. coli*). It is indicated for the treatment of congenital and acquired neutropenia such as in chemotherapy-receiving cancer patients as well in HIV patients[27–29]. Administration of filgrastim has demonstrated improved survival in congenital neutropenia patients[30]. In acquired neutropenia *i.e.* chemotherapy, accelerated recovery of neutrophils and reduced duration of neutropenia have been seen during randomized trials[28]. In addition, filgrastim is usually administered in bone marrow donors prior to harvesting to increase the number of peripheral blood stem and progenitor cells[31]. At present, there are several authorized biosimilar filgrastim products in the EU with good safety track records (**Table 2**).

Table 2. List of Innovator and Biosimilar Filgrastim Products on The EU Market

Brand	Host cells	Ref.
Innovator filgrastim		
Neupogen® (Amgen Inc.)	*E. coli*	32
Biosimilar filgrastim		
Filgrastim Hexal® (Hexal AG)	*E. coli*	33
Zarzio® (Sandoz GmbH)		34
Biograstim® (AbZ-Pharma GmbH)	*E. coli* K802	35
Ratiograstim® (Ratiopharm GmbH)		36
Tevagrastim® (Teva GmbH)		37
Nivestim® (Hospira UK Ltd)	*E. coli* BL21	38
Grastofil® (Apotex Europe BV)	*E. coli* BL21	39
Accofil® (Accord Healthcare Ltd)		40

The "true" definition of biosimilars

There is no scientific definition of a biosimilar. Biosimilar defines a product that has been approved by a regulatory agency based on biosimilar regulations requesting comparative clinical data. To date, the EMA has authorized the most biosimilars around the globe under the biosimilar guidelines. These guidelines were more or less copied by other countries such as the US[41,42]. In 2015, the US FDA approved the very first biosimilar, *i.e.* Zarxio[43]. The WHO has also established the Similar Biotherapeutic Product (SBP) guidelines, which adapt the EU regulatory requirements. These are the basic level of regulation, especially for third world countries[44]. In addition to these "true" biosimilars, copies of innovator biotherapeutics that have not been approved through a stringent regulatory process are available and are posing major challenges in the developing world.

A good illustration is the increase in epoetin–associated pure red cell aplasia (PRCA) cases among Thai patients[45,46]. The upsurge of this adverse immunological effect was observed by many clinicians following the increased use of copy epoetin products for economic reasons. In contrast to the EU biosimilar paradigm, these copies were approved by the Thai FDA under the generic paradigm with no comparative clinical data required[47]. As indicated by some studies, while several factors may contribute to the increase in PRCA, including handling and storage[48] and the genetic background of the patient population[49], one important factor could be the inferior quality of these non-regulated copies[50].

The wind of change in biosimilarity's practice

There is no consensus within the medical community about the need for clinical data to show clinical equivalence for biosimilars. On one hand, some groups are convinced that more and longer safety data of biosimilars is required to justify possible differences in clinical effects[51,52]. On the other hand, some experts propose to drop the clinical comparability exercise of biosimilars, believing that in recent years knowledge about manufacturing as well as characterizing proteins and the vast experience in assessing biosimilars by manufacturers and regulatory authorities have

increased dramatically and sufficiently[53]. Further, one expert has pointed out that running extra trials could be unnecessary and unethical, and contrary to the intent that biosimilars are trying to achieve, i.e. a cheaper alternative[54].

In addition to doubt concerning the added value of clinical trials, there is also some winds of change for other than regulatory reasons. To begin with, US institutional investors have urged more than two dozen drug makers and biotech firms not to denigrate biosimilars[55]. Their argument is mainly that biosimilars will lower drug prices. At the same time, drug maker boards have recently sought to downplay the patient safety record of EU biosimilars and have requested different International Non-Proprietary Names (INN) for biosimilars. Such actions are seen by the investors as a potential hindrance for the acceptance of biosimilars in the US market, thereby forestalling any projected savings. The investors have pointed out that experience in the EU thus far with marketed biosimilars has been noticeably free of immunogenicity events and that biosimilars can still be safely tracked despite having INNs identical to those of innovator products[56].

Furthermore, the request for distinguishable INNs has also been criticized outside the drug industry[57-59]. For example, the editorial team of Nature Biotechnology's journal see this request as market driven and meant to give biosimilars a bad name[60]. They contend that if different INNs are assigned to biosimilars, biotherapeutics produced by different processes would also need different INNs. This view is shared by an expert in the field, who added that sharing the same INN for innovator biotherapeutic products with different labelling has never been an issue before[61].

The WHO also plays a role in the evolution of the biosimilars' regulatory landscape. During the sixty-seventh World Health Assembly, access to biotherapeutics including biosimilars was on the agenda[62]. It was agreed that the next SBP guidelines should also include affordability as a major consideration for biotherapeutics including biosimilars, while still ensuring their quality, safety and efficacy[63].

In addition, the Colombian Ministry of Health and Social Protection has taken further measures to promote affordability by releasing a decree on the registration process for biotherapeutics[64]. The decree includes an abbreviated third route. In contrast to the first two routes i.e. completeness and comparability routes, the abbreviated route may be used for proposed products that are deemed adequately described under either of these two circumstances: 1) no substantial differences in term of product's identity, biological activity, physiochemical properties, and purity from the innovator product as claimed by the manufacturer or 2) its active substance is included in a pharmacopoeial monograph. Further justification, such as comparative quality and clinical data, are not required. In addition, any clinical evidence from specified health authorities abroad indicating that the active substance has well-documented safety and efficacy profiles based on considerable clinical experience and robust safety information may be used, thereby improving affordability by reducing development cost[65].

Also, EMA/CHMP is increasingly viewing clinical trials as in general insensitive to differences between biosimilar and reference products. This is reflected in, for instance, the draft regulatory authorities have made of a concept guidance to waive the clinical studies of G-CSF[66].

Currently, the development of biosimilars is still a lengthy and expensive process. Four development stages contribute costs, each with varying requirements and taking varying amounts of time. Execution of a clinical trial is still the major cost driver for biosimilars[67]. Because clinical

trials are mandated by the regulators to demonstrate that quality differences are not clinically meaningful, the nature and extent of a clinical trial will depend on the outcomes of the comparability quality studies. Unless the link between quality attributes and potential clinical outcomes can be established, producing affordable biosimilars will continue to face these hurdles.

AIM

The overarching aim of this thesis is to link quality attributes of biotherapeutics to their potential safety issues including immunogenicity, by comparing quality of biosimilars and/or copy biotherapeutics to each other as well as to innovator products and relating these comparative quality data to clinical data. Based on the outcome of these studies, recommendations to the Thai FDA on how to deal with non–regulated copy epoetin products currently licensed through the generic pathway will be provided.

OUTLINE OF THIS THESIS

This thesis covers the physiochemical and biological characteristics of high numbers of epoetin and filgrastim innovator and biosimilar products approved in the EU as well as copy biotherapeutic products from other regions, such as Thailand. Subsequently, the quality data of non–regulated copy of epoetin products procured from the Thai drug market are linked to clinical data of Thai patients treated with these products.

In **Chapter 2** we review any existing evidence on biosimilar products–specifically safety issues, including immunogenicity – to investigate the added value of biosimilarity exercise beyond quality.

In **Chapter 3** we analyze the quality and batch-to-batch consistency of innovator filgrastim, Neupogen®, as well as two biosimilar filgrastim products authorized in the EU via the biosimilar pathway, Zarzio® and Tevagratim® and two copy filgrastim products licencesed in less regulated markets through local regulatory approval processes, Biocilin® and PDgrastim®.

In **Chapter 4** we assess the quality and batch-to-batch consistency of different innovator, Eprex® and NeoRecormon®, and biosimilar epoetin products, Binocrit® and Retacrit®, currently marketed in the EU.

In **Chapter 5** we perform a comparability study covering the physiochemical and biological characterization of 12 copy epoetin products that are available on Thai drug market compared to the innovator product, Epogen®.

In **Chapter 6** we present the clinical data from the prospective immunogenicity surveillance registry of epoetin with subcutaneous exposure in Thailand and the quality assessment of copy epoetin products identified to be involved with PRCA cases.

In **Chapter 7** we summarize our findings and provide perspectives on the changing landscape of biosimilars.

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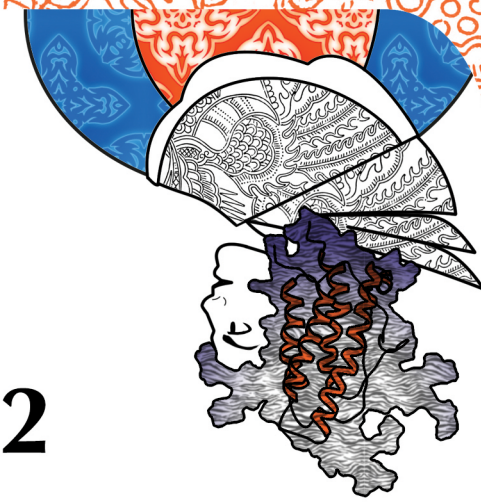

Chapter 2

Who needs biosimilarity data beyond quality?

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ABSTRACT

The biosimilar pathway differs from the generic classical pathway. This raises, in particular, a need to demonstrate clinical similarity between biosimilar and reference. Although clinical comparability exercises give reassurance that a proposed biosimilar will have safety and efficacy profiles comparable to the reference, it has also been challenged to be too cautious and may hinder the development of subsequent generations of biosimilars. Here, we review the evidence base for safety issues surrounding biopharmaceuticals, to contribute to the debate regarding the value of biosimilarity beyond quality.

INTRODUCTION

In 2004, the European Medicines Agency (EMA) pioneered the development of the biosimilar pathway for biopharmaceuticals, laying down the requirements needed to demonstrate similarity to an authorized reference product[1]. Unlike the classical generic pathway, the biosimilar pathway requires comparative clinical efficacy studies, because the analysis of only physicochemical properties and biological activity cannot fully predict clinical outcomes. Since 2006, the EMA has approved biosimilar versions of insulin glargine, somatropin, epoetin, filgrastim, follitropin alfa, infliximab and etarnecept[2]. Thus far, biosimilar specific safety and immunogenicity issues either have not been reported for any of the marketed biosimilars in the EU or were detected before the biosimilar reached the market *e.g.* HX575[3]. This gives reassurance that the EU tailored biosimilar pathway is enabling the introduction of efficacious and safe products.

The EU regulatory framework is in continuous development and several guidelines have been updated since their initial adoption. The EMA has recently released a concept paper to revise the clinical requirements for granulocyte colony-stimulating factor (G-CSF), thereby trying to establish criteria to waive clinical studies[4]. This comes as no surprise, as earlier guidelines hinted comparative clinical trials are not always mandatory "*In exceptional cases, the confirmatory clinical trial may be waived if physicochemical, structural and in vitro biological analyses and human pharmacokinetic (PK) studies together with a combination of pharmacodynamics (PD) markers that reflect the pharmacological action and concentration of the active substance, can provide robust evidence for biosimilar comparability*"[5]. In line with the EMA, the US Food and Drug Administration (FDA) has released guidance of clinical pharmacological data to support a demonstration of biosimilarity to a reference product, indicating the possibilities to perform only selected clinical studies when comparative analytical characterization indicates "highly similar proposed biosimilar with fingerprint-like similarity"[6].

Nonetheless, current practice in granting marketing authorization of biosimilars still heavily relies on clinical studies. These regulatory demands for clinical studies have recently been challenged as being too cautious and hindering the development of subsequent generations of biosimilars[7]. At the same time uncertainty remains, and some prescribers are demanding more and longer clinical studies to establish the safety and efficacy of biosimilars[8-10].

To contribute to the debate regarding the added value of biosimilar comparability exercises beyond quality, we have reviewed the evidence base for the unpredictability of safety issues of biopharmaceuticals.

The quality assessment of biosimilars

A biosimilar is developed using reverse engineering. The specifications of the biosimilar are determined through extensive analyses of the structure and physical chemical and biological properties of multiple batches of the reference product. In the EU, the reference product for a proposed biosimilar must be a product that has already been authorized locally or at least within International Conference on Harmonization countries on the basis of a complete dossier[11]. **Table 1** lists the generally assessed quality attributes as recommended by the regulatory guidelines[12-14]. Subsequently, the production and downstream processes are developed and, if necessary, adapted

to obtain a product meeting those specifications. Only then can the biosimilar manufacturer proceed to pre-clinical and clinical studies, the design of which is determined by the level of similarity in structure and by physiochemical as well as biological characteristics.

Table 1. Quality attributes generally observed in biopharmaceuticals

Parameters	Quality attributes
Physicochemical properties	amino acid sequence free sulfhydryl (SH) group(s) and disulfide bridges N- and C-terminal amino acid sequence primary and higher order structure glycan profile site-specific glycosylation patterns site occupancy
Biological activity	potency ligand neutralization Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Complement Dependent Cytotoxicity (CDC) apoptosis
Immunochemical properties	specificity binding affinity to target antigen and relevant receptors binding kinetics Fc functional activity
Purity and impurities	high–molecular weight species incl. aggregation enzymatic post-translational modifications (PTMs) *i.e.* glycosylation and phosphorylation non-enzymatic PTMs includes oxidation, deamidation/isomerization, truncation and glycation (sub)visible particulates charge variants
Process-related impurities and contaminants	bacterial endotoxins host cell proteins residual DNA
Quantity	protein concentration (content)

Within the EU, several analytical methods and required specifications for biopharmaceuticals of five different product classes can be found in the monographs of European Pharmacopeia (Ph. Eur.); these are summarized in **Table 2**. In addition, international standards and reference preparations established for a wide range of biopharmaceutical substances prepared using recombinant DNA technology are available from several custodian laboratories of World Health Organization, including the European Directorate for the Quality of Medicines and Healthcare and the National Institute for Biological Standards and Control. These standards and materials can be used to calibrate methods of analysis or to calibrate in-house standards but are not intended to stand in for a reference product during a biosimilar comparability exercise.

Biosimilarity beyond quality? Or can you predict safety and efficacy from the quality data?

The requirement for clinical trials for a biosimilar prior to regulatory approval is based on the assumption that clinical equivalence cannot be assumed based on physicochemical and biological similarity alone. The nearly exclusive argument for this assumption is the severe side effect that appeared in 1998 after a slight formulation change was introduced in Eprex®, an epoetin alfa marketed by Johnson & Johnson (J&J). This formulation change had been accepted on the basis of physicochemical characterization and a PK study. No immunogenicity study was required as no anti-drug antibodies (ADAs) against epoetin had been reported. And there was no reason to believe that a formulation change would induce immunogenicity. Unexpectedly, more than 275 chronic renal failure patients developed high levels of neutralizing antibodies to epoetin, leading to a severe anemia (Pure Red Cell Aplasia, PRCA) by cross neutralization of endogenous epoetin[20]. This problem was eventually countered by contraindicating subcutaneously (SC) use in chronic renal failure patients and possibly by changes made to the product in terms of a rubber stopper coating.

On the basis of their root-cause analyses, J&J suggested that the increased immunogenicity was related to an adjuvant effect of leachables derived from the uncoated rubber stopper, although this has been criticized[22]. The most likely explanation of increased immunogenicity of Eprex was a less stable formulation and consequently aggregation when the product was improperly handled[21].

There are other arguments for aggregation as the cause of immunogenicity of epoetin. A good example is HX575, an epoetin alfa biosimilar. Two patients developed neutralizing antibodies during a clinical trial of SC administered HX575[3]. High levels of protein denaturation and aggregation caused by tungsten introduced during the manufacture of the syringes was the likely cause of the increased immunogenicity[22]. Another example is the increased PRCA incidence associated with intended epoetin copy products reported in Thailand[23]. Recent studies have associated an increase in PRCA with the presence of protein aggregation[24]. And aggregation as the cause of immunogenicity of epoetins fits with immunogenicity of other biopharmaceutical products i.e. interferons, growth hormone (GH) and insulin, which was also found to be related to aggregates[25-28].

When J&J introduced the new formulation of Eprex in 1998 they noted a higher level of aggregates. It was reported to be within specifications, but specifications are in general not defined on the basis of biological effects[29]. And the link between immunogenicity and aggregates was not well established in 1998. If a higher level of aggregation in epoetin were to be reported now, it would never be authorized by regulatory authorities without a proper evaluation of a possible increased risk of immunogenicity. Hence, it is highly unlikely that the Eprex problem will ever be repeated.

Current experience thus shows that the immunogenicity of biopharmaceuticals is a complex process influenced by many factors[30]. The primary factor is the product[31]. The two product components that can initiate immunogenic response are the structure of the molecule (intrinsic immunogenicity) and/or its impurities (extrinsic immunogenicity). Examples of products with a high level of intrinsic immunogenicity because of the presence of neo-epitopes include non-human therapeutic proteins like bacterial asparaginase and animal derived antisera[30]. The best studied impurities responsible for extrinsic immunogenicity are aggregates[27].

Table 2. List of quality attributes need to be assessed based on Ph. Eur. monographs edition 8.8

	Somatropin (0950)	Epoetin (1316)	Filgrastim (2206)	Follitropin (2286)	Insulin glargine (2571)
Identification					
A. protein content and/or biological activity	√	√	√	√	√
B. molecular weight (or size) variants	√	√	√	√	
C. isoforms	√	√	√	√	
D. product-related substances and impurities	√	√	√		
E. protein structural characterization and confirmation	√	√	√	√	√
F. additional		N-terminal sequence		carbohydrate structure	
Tests					
protein concentration		√			
molecular size variants i.e. dimers and/or oligomers	√	√	√	√	√
molecular mass variants			√	free subunits content	
charged variants	deamidated forms		√		
others		sialic acid content			zinc content water content
product-related substances and impurities	desamido-somatropin		oxidized form reduced form	oxidized form	O^A-Arg-insulin glargine
Endotoxin	√	√	√	√	√
Assay					
protein content	√		√	√	√
potency (biological activity)		in vivo bioassay	in vivo bioassay	in vivo bioassay	
Ref	15	16	17	18	19

Predicting immunogenicity of a new human therapeutic protein is difficult[32]. Immunogenicity cannot be fully predicted even in studies involving non-human primates[33]. However in the case of biosimilars, the question is relative immunogenicity in comparison with the reference product. Because regulators expect an identical amino acid sequence and structure between biosimilar and reference product, no differences in intrinsic immunogenicity are to be expected. If no differences in impurity profiles between biosimilar and reference are identified, differences in immunogenicity are very unlikely.

The unpredictability of the clinical effect, based on the quality of the product, is also contradicted by the experience with the manufacturing changes of marketed biopharmaceuticals. Considering the number of products on the market and the constant product improvements, hundreds of manufacturing changes are introduced each year[34]. As far as we know, the Eprex–PRCA incident, more than 18 years ago, is still the only example of what was then considered an unpredictable side effect. The only case with any resemblance is the hypersensitivity reactions in some patients receiving Cetuximab®, a mAb directed against the human epidermal growth factor receptor, which were related to glycosylation *i.e.* galactose–α–1, 3–galactose (α-Gal) residues in the product's fragment antigen-binding domain[35]. α-Gal residues are mostly expressed at the terminal side, thereby causing hypersensitivity reaction particularly in patients with pre-existing anti alfa-Gal antibodies[36,37]. Even so, this hypersensitivity reaction can be considered predictable.

Depending on the type of manufacturing change, regulators demand biological or clinical testing to exclude unexpected responses in patients. Several examples in the public domain show changes in clinical properties after changes in manufacturing. For example, differences in PK of Raptiva® (Efalizumab) manufactured at two different sites (XOMA and Genentech) were linked to the differences in the C-terminal processing, acidic forms and galactose[38]. Subsequently, differences in PKs between myozyme produced in 2000 L tanks and in 160 L tanks was associated with differences in the carbohydrate structures of their molecules[39]. Exploratory analyses of the presence of anti-recombinant human acid alpha-glucosidase IgG antibody in patients with late-onset Pompei disease also suggest higher immunogenicity for the 2000 L product than for the 160 L product[40]. In both cases there is a clear link between quality and the clinical reactions.

The development of biosimilars is also yielding convincing evidence about the relation between physicochemical properties and clinical behavior. In **Table 3**, the differences between current biosimilars and their reference products are shown as quality attributes. No biosimilar specific safety issues have yet been identified, and the clinical differences seen during development could always be related to clear quality differences.

For instance, observed clinical differences between Retacrit®, a biosimilar epoetin alfa, and its reference, Eprex, were associated with differences in potency[41]. Despites the same stated dose, a 10% higher mean weekly dose of Retacrit was required to correct hemoglobin (Hb) values during the correction study. In the maintenance study, following a switch from Eprex to Retacrit, the required dose was elevated to approximately 10–15% with a decrease of Hb level of 5%. The opposite scenario saw a 10% increase in Hb level, with a 10% decrease in the administered dose. Upon repeated testing, the apparent lower potency of Retacrit reflected the fact that Eprex had a higher that labeled potency. This higher-than-declared potency of Eprex was also indicated in another study[24].

Another clinical issue was picked up during two phase III clinical trials in children with GH deficiency receiving somatropin (Omnitrope®) Sandoz powder manufactured by Covance[42]. Due to high concentrations of host cell protein (HCP), up to 60% of the enrolled patients tested positive for anti-GH antibodies and all patients were tested positive for anti-*Escherichia coli* antibodies. Upon the introduction of additional purification steps, the level of HCP was reduced to the acceptable range known from other authorized GH-containing products and so was the level of anti-GH antibodies.

Based on years of manufacturing and clinical experience with biopharmaceuticals, several quality attributes that have impacted immunogenicity have been identified. The acceptance criteria of these critical attributes needs to be based on a full characterization of the reference product together with an understanding of the variability and consistency of the reference product attributes. That said, there is nothing else needed for predicting immunogenicity of biosimilars besides quality data.

Other safety issues with biopharmaceuticals are associated with PD effects. Even so, if potency of the biosimilar and reference are comparable, both will have comparable efficacy and safety profile. To test for potency, there are often *in vitro* assays available. For the rest, potency can be measured by either animal or human PD studies. Only in rare cases have tests for potency required studies in patients with a clinical endpoint.

A further issue with biopharmaceuticals is skin reactions. In general, these are formulation-associated. In case of identical formulation between biosimilars and the reference, skin reactions are unexpected. Only a biosimilar using a new formulation warrants additional studies to evaluate potential safety issues.

Table 3. Accepted quality differences of currently approved biosimilars compared to the reference products as based on the EPAR.

Biosimilar product	Reference product	Differences in physiochemical and biological characteristics			
		Formulation	Impurities	Glycosylation	Potency
Abasaglar	Lantus	√	√		
Omnitrope	Genotropin		√		
Retacrit and Silapo	Eprex			√	
Binocrit, Epoetin alfa Hexal and Abseamed	Eprex		√	√	
Accofil and Grastofil	Neupogen				
Biograstim, Ratiograstim and Tevagrastim	Neupogen		√		
Zarzio and Filgrastim Hexal	Neupogen	√			
Nivestim	Neupogen				
Bemfola	Gonal-f	√		√	
Ovaleap	Gonal-f			√	
Remsima and Inflectra	Infliximab	√	√	√	√
Benepali	Etarnecept		√	√	√

CONCLUDING REMARKS

Biopharmaceuticals differ from small molecules drugs in many respects, one of which is that nearly all of them are capable of inducing the formation of ADAs in patients. Because of the notion that physicochemical and biological characteristics are insufficient to predict clinical outcomes, clinical studies are still mandated by regulators, *i.e.* the EMA, to confirm similarity in efficacy and safety profile, including immunogenicity.

Nonetheless, we question the significance of biosimilarity beyond quality in precluding unexpected immunogenicity in patients. Based on the evidence presented here, differences in immunogenicity are likely related to the extrinsic immunogenicity of biosimilars. This suggests that there is nothing else needed beyond quality data to predict similarity in immunogenicity between biosimilar and reference product.

Analytical tools have advanced tremendously, allowing evaluation of nearly all quality attributes at much greater sensitivities. Integration of data from multiple analytical and biological tests provides complete understanding of the level of similarity in quality between two biopharmaceutical products even for the class of monoclonal antibodies. Furthermore, the EMA is already exploring the possibility to establish criteria waiving confirmatory clinical trials for biosimilars, starting with G-CSFs. Although these changes are encouraging and potentially can be applied for other classes of biosimilars, for products where risk analysis suggests either the formation of ADAs may cross-react to and inhibit endogenous factors *e.g.* PRCA or may neutralize treatment in which there is no alternative treatment such as with enzyme replacement therapy, clinical studies will still be needed to monitor potential safety concerns.

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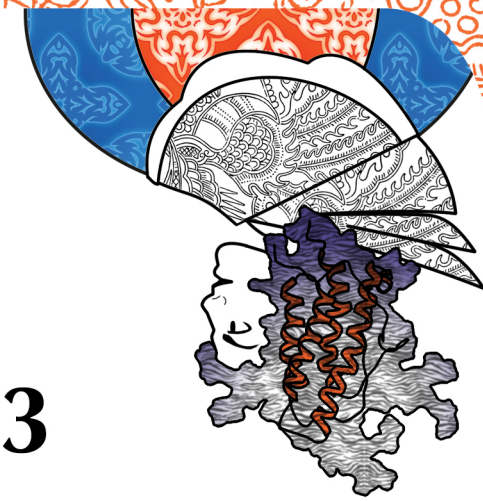

Chapter 3

Quality Comparison of Biosimilar and Copy Biologic Filgrastim Products with the Innovator Product

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ABSTRACT

Background: Filgrastim is a recombinant human granulocyte-colony stimulating factor (rhG-CSF) widely used to treat congenital and acquired neutropenia. Biosimilar filgrastim products have been approved in the EU and shown to be comparable with the innovator with respect to quality, safety and efficacy. In less regulated markets, copy filgrastim products are available but data regarding their quality are scarce.

Objective: To provide head-to-head comparative quality studies of biosimilar and copy filgrastim products.

Methods: Innovator filgrastim product, Neupogen®, two EU-licensed biosimilar filgrastim products: Zarzio® and Tevagrastim®, and two copy filgrastim products: Biocilin® and PDgrastim®, were subjected to (1) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, (2) high–performance size–exclusion chromatography, (3) reversed–phase ultra performance liquid chromatography, (4) micro-flow imaging, (5) endotoxin test and (6) *in vitro* potency testing.

Results: Zarzio® and Tevagrastim® have comparable quality compared to Neupogen®, while Biocilin® showed a significantly lower and PDgrastim® a higher specific activity.

Conclusion: Except for the specific activities of the two copies we found no clinical significant differences in product quality between the filgrastim products studied.

KEYWORDS recombinant human granulocyte-colony stimulating factors, filgrastim, biosimilar, copy biologics, physicochemical properties, protein characterization

INTRODUCTION

Recombinant human granulocyte-colony stimulation factor (rhG-CSF) is one of the first recombinant biologics authorized for use in hematology[1]. Two different forms of rhG-CSF are distinguished, namely filgrastim (from *Escherichia coli* [*E. coli*], under the trade name Neupogen®) and lenograstim (from Chinese hamster ovary cell, brand name Granocyte®)[2]. Filgrastim contains 175 amino acids and differs from the endogenous form in that it lacks O-glycosylation and has an additional methionine group at the N-terminal[3]. Its use was approved in 1991 both in the EU and US. A next generation filgrastim is the PEGylated long circulating pegfilgrastim (brand name Neulasta®). Owing to the addition of 20 kDa polyethylene glycol (PEG) to the N-terminal methionyl residue of filgrastim, pegfilgrastim has an extended circulating half-life, and thus can be administered less frequently than filgrastim[4]. Rh-GCSF is commonly prescribed to treat both congenital and acquired neutropenia such as in patients undergoing chemotherapy or patients diagnosed with advanced AIDS[5-7]. It is characterized by a low neutrophil count, making patients more prone to bacterial infections. In addition, rhG-CSF is usually administered in donors of peripheral blood stem and progenitor cells prior to harvesting[8].

Patent expiration of innovator biologic products has created the possibility for companies to develop biosimilars. Within the EU, biosimilars are authorized via an abbreviated regulatory pathway, which requires demonstration of similarity in terms of quality, safety and efficacy, to an innovator product already licensed in the EU[9]. Many countries are introducing specific biosimilar regulations using the European approach as guidance, adapted to local needs and demands[10]. In some regions, copy versions of biologics predate the existence of biosimilar guidelines. These have been termed 'non-original biologics" or "non-innovator biologics"[11].

Biologics, as opposed to chemically-synthesized small molecule drugs, are extremely intricate. The characterization of biologics is a challenge. The use of living cells may introduce subtle differences despite using the same gene sequence and vector as the innovator biologics. Further, all biologics are subject to post-translational modifications such as acetylation and oxidation, which may affect protein function and result in heterogeneity.

Product-related quality differences may in principle lead to serious adverse events. An often cited incidence of this occurred in 1998 when a minor change in the formulation of recombinant human erythropoietin elicited the development of anti-drug antibodies, neutralizing endogenous erythropoietin and leading to pure red cell aplasia in renal failure patients[12]. This is an indication of how subtle changes in the manufacturing process may have unforeseen clinical consequences.

Currently, seven biosimilar filgrastim products have been approved in the EU[13]. One product has successfully entered the US market as the first US biosimilar: Zarxio®[14]. All demonstrate comparable quality, safety and efficacy to Neupogen® as reported in several comparative studies[1,15-18]. In less regulated markets, various other copies of filgrastim products have been identified. In India, several copy filgrastim products show similarity to Neupogen® with respect to physicochemical and biological characteristics[19,20]. In Egypt, the quality of one copy filgrastim product was reported to be inferior to the innovator product[21]. However, there is scarcity of data regarding direct comparison between the biosimilar and copy filgrastim products and the innovator product. The perception of clinicians is that copy biologics from less regulated markets, such as

parts of South America, are inferior to biosimilars as these products have not been approved through a stringent regulatory process[22].

METHODS

Visual inspection

Table 1 lists innovator, biosimilar and copy filgrastim products procured for our research. Different batches of Neupogen were either provided by Sandoz or purchased from pharmacy at University Medical Centre Utrecht (UMCU), Utrecht, The Netherlands. Biosimilar filgrastim products, Zarzio and Tevagrastim, were provided by the pharmacy of the UMCU. Copy filgrastim products, Biocilin and PDgrastim were locally procured from Mexico and Iran, respectively, and shipped to Utrecht University. All products were stored at between 2-8°C and handled according to the manufacturers' specifications. Prior to any measurements, all formulations were visually assessed at the lab bench to check for the presence of visible particulates. Expired vials of Neupogen and PDgrastim were analyzed in current study to gain insight into physicochemical and biological characteristics of filgrastims in marketed products as worst case scenario.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed under non-reducing and reducing conditions using Bolt® 4–12% Bis-Tris Plus Gels (10 wells), installed on Bolt® Mini Gel Tank. On each well, 50 ng of protein was loaded upon mixing with 1x Bolt® LDS Sample Buffer and heated for 5 min at 95°C. Under reducing condition, 1x Bolt® Sample Reducing Agent was added. PageRuler Plus Prestained Protein Ladder, 10–250 kDa was used as a molecular weight marker. Filgrastim chemical reference substances (CRS) was commercially available from the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France) and used as a control. The running condition was set to constant 165 Volt for 45 min in 1x Bolt® MOPS SDS running buffer. Proteins were visualized using SilverQuest™ Silver Staining Kit according to the manufacturer's specifications. All materials were ordered from Thermo Scientific (Landsmeer, The Netherlands).

High–Performance Size–Exclusion Chromatography (HP–SEC)

Following the European Pharmacopoeia (Ph. Eur.) monograph for filgrastim concentrated solution[23], HP-SEC was performed on a 5 µm, 300 x 7.8 mm TSKgel® G3000SW$_{XL}$ column (Tosoh Bioscience, Griesheim, Germany) with SecurityGuard Universal Guard Cartridge System as a pre-column (Phenomenex, Utrecht, The Netherlands). Both pre-column and column were installed in a Waters 2695 Separations Module (Waters Corporation, Milford, Massachusetts, USA) equipped with Waters 2487 Dual λ Absorbance UV and Waters 2475 Multi λ Fluorescence detectors. UV detection was performed at 215 nm and 280 nm. For fluorescence detection, excitation was performed at 280 nm, and the emission was recorded at 340 nm. A mobile phase containing 50 mM ammonium hydrogen carbonate (Sigma-Aldrich, Zwijndrecht, The Netherlands), pH 7.0 adjusted with phosphoric acid (Acros Organics, Geel, Belgium) and filtered through 0.45 µm

nylon filter (Sartorius Stedim, Göttingen, Germany). Prior to injection in triplicate, all filgrastim products were diluted to a concentration 0.25 mg/mL in a dilution buffer of 100 mM sodium acetate, pH 5.0, adjusted with acetic acid (Merck, Darmstadt, Germany). Separation took place at a flow rate of 0.5 mL/min for 30 min isocratically. At the beginning of each experiment, 50 µL of 1 mg/mL bovine serum albumin (Sigma-Aldrich) in dilution buffer was injected into the column to reduce non-specific adsorption. The temperature of the sample maintained at 20°C and the column at 30°C. A calibration curve was constructed using seven concentrations of filgrastim CRS in the range of 0.05–1 mg/mL and used to calculate the concentration of filgrastim monomer in all products (peak areas vs. concentration). All analysis was performed in Empower 3 software version 7.21.00.00.

Table 1. List of Tested Filgrastim Products

Trade name (company)	Lot. No.	Declared potency	Declared content	Excipients	Presentation
		Analyzed within expiry date			
Neupogen® (Amgen)	1042036A	30 MU/0.5 mL	300 µg/0.5 mL	Acetate, sorbitol, polysorbate 80 (PS80)	PFS
Zarzio® (Sandoz)	46081101	30 MU/0.5 mL	300 µg/0.5 mL	Glutamate, sorbitol, PS80	Vial
Tevagrastim® (Teva Pharma)	FL5028G	30 MU/1.0 mL	300 µg/0.5 mL	glacial acetic acid, sorbitol, PS80	PFS
Biocilin® (Dong-A-Pharmaceutical)	40168	Not declared	300 µg/1.2 mL	Not stated	Vial
		Analyzed past expiry date			
Neupogen® (Amgen)	1025277 (A)	48 MU/1.6 mL	480 µg/1.6 mL	Acetate, sorbitol, polysorbate 80 (PS80)	Vial
	1026361 (B)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
	1026690 (C)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
	1026689 (D)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
	1027991 (E)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
	1028686 (F)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
	1028687 (G)	48 MU/1.6 mL	480 µg/1.6 mL		Vial
PDgrastim® (PooyeshDarou)	90034	30 MU/0.5 mL	300 µg/1.0 mL	Sodium acetate	Vial

PFS, pre-filled syringes

Reversed–Phase Ultra Performance Liquid Chromatography (RP–UPLC)

RP-UPLC was performed on an Acquity® Ultra Performance LC system (Waters Corporation) where a UPLC Acquity BEH300 C4 column (1.7 µm, 2.1 x 50 mm) was installed. Column was equilibrated at 60°C with 85% mobile phase A (0.1% trifluoroacetic acid [TFA, Sigma-Aldrich] in MilliQ water) / 15% mobile phase B (0.1% TFA in 90% HPLC grade acetonitrile [Biosolve, Valkenswaard, The Netherlands]) until stable baseline was reached. Separation was achieved by applying linear gradients: 15% B (0–0.5 min), 15% B–75% B (0.5–10.5 min), 75% B–15% B (10.5–11 min) and 15% B (11-11.5 min) at a flow rate of 0.25 mL/min. While being maintained at

20°C, 1.5 µg of each filgrastim products was injected in triplicate.

Oxidized forms were optimized from Ph. Eur. monograph for filgrastim concentrated solution where an aliquot of 100 µL of 0.5 mg/mL filgrastim CRS was treated with 3 µL of 30% hydrogen peroxide (Merck) and incubated at 25°C for 15 min. Reduced forms were similarly produced as described on Ph. Eur. monograph for filgrastim concentrated solution[23]. Detection took place with an Acquity™ PDA detector (Waters Corporation) at 215 nm.

Endotoxin Test

Endotoxin concentration in filgrastim products was quantified with endpoint chromogenic Limulus amebocyte lysate (LAL) QCL1000™ assay as described by the manufacturer (Lonza, Basel, Switzerland). Briefly, each filgrastim product was diluted in LAL reagent water at ratio 1:10, 1:20 and 1:40. Twenty five of each dilution transferred to each well of prewarmed LAL reagent grade multiwell plate at 37°C in duplicate. Hereafter, an equal volume of LAL was added to each microplate well followed by gently tapping on the side to facilitate mixing, and further incubated at 37°C for another 10 min. Into each well, 50 µL of chromogenic substrate was then dispensed. Reaction was stopped after 6 min by adding 50 µL of 25% (v/v) acetic acid (Sigma-Aldrich). Absorbance was read at 405 nm by a SPECTROstar® *Nano* (BMG Labtech, Ortenberg, Germany) plate reader. A calibration curve was included by preparing dilution series of *E. coli* 0111:B4 Endotoxin (0.011 EU/mL). Eventually, the endotoxin concentration of filgrastim products was determined from their absorbance by linear regression. Additionally, to verify the lack of product inhibition, dilutions of filgrastim products were spiked with a known amount of endotoxin *i.e.* 0.4 endotoxin units (EU)/mL. All reagents and materials used are purchased via Lonza and endotoxin free grade-certified, unless indicated otherwise.

Micro–Flow Imaging (MFI)

The analysis of (non-) proteinaceous sub-visible particles was performed on MFI5200 instrument (Protein Simple, California, USA) equipped with a saline-coated 100 µm flow cell. MFI was operated at high magnification (14x) and controlled using the MFI View System Software version 3.1. Between each measurement, MFI system was flushed with 2 mL MilliQ water at flow rate 0.7 mL/min and checked for a clean background. Formulation buffer (50 mg/mL sorbitol, 0.04 mg/mL Tween 80, 0.59 mg/mL sodium acetate, and 0.035 mg/mL sodium chloride) at flow rate 4 mL/min was filtered through 0.2 µm filter (Sartorius Stedim) and used to "optimize illumination" prior to evaluating each sample. Due to limited volume, only a few products were analyzed once at a sample volume of 500 µL. Data analysis was performed in MFI View Analysis Suite version 1.4. Air bubbles, if any, were excluded from analysis by applying filters "Intensity Min" and "Aspect Ratio" as described elsewhere[24]. All chemicals were purchased from Sigma-Aldrich.

In Vitro Potency Assay

An *in vitro* potency assay was performed based on the proliferation of M-NFS-60 cells induced by G-CSF as described on Ph. Eur. monograph for filgrastim concentrated solution[23]. In short, all filgrastim products and the World Health Organization (WHO) 2nd International Standard (IS) for human G-CSF (NIBSC, Hertfordshire, UK) were prepared in assay medium (RPMI 1640 supplemented with Lglutamine, sodium bicarbonate, 10% Fetal Bovine Serum, 10 mM HEPES buffer and 0.05 mM 2-mercaptoethanol) at a starting concentration 20 ng/mL and were added to each well of 96-well microtiter plate (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) in triplicate. A series of 17 threefold dilutions was subsequently prepared to obtain a standard curve. M-NFS-60 cells were harvested and washed twice in assay medium. Hereafter, ~20,000 cells were added to each well.

After being incubated for 44–48 hours at 37°C with 5% CO_2, the cell proliferation was quantified. CellTiter 96® AQueous One Solution Cell Proliferation Assay solution (Promega, Leiden, The Netherlands) was added to the cells and the plate was re-incubated for 4 hours. The quantity of formazan produced was estimated by recording the absorbance at 490 nm and 650 nm (reference wavelength) with a SPECTROstar® *Nano* microplate reader (BMG Labtech). *In vitro* potency in all filgrastim products was calculated based on WHO 2nd IS with an assigned value for G-CSF activity of 95,000 IU/ampoule using the parallel line assay in Combistats software version 5.0 (EDQM)[25].

RESULTS

Prior to characterization, all filgrastim products were examined visually. All products were clear and colorless solutions and contained no visible particulates.

Characterization of impurities with molecular mass differing from that of filgrastim

The identity and purity of different filgrastim products were assessed by SDS–PAGE and HP–SEC. **Figure 1** exhibits silver stained SDS–PAGE gel of all products with a principal band in the range of 15-25 kDa under both non-reducing and reducing conditions. As is the case with the control (image not shown), this band corresponds to the monomeric filgrastim with theoretical molecular weight of 18.8 kDa[15]. No other bands at higher or lower molecular weight than filgrastim monomer were detected in all products including expired vials. Faint bands identified in some lanes at between 10 and 15 kDa were likely due to overloading of pre-stained protein marker.

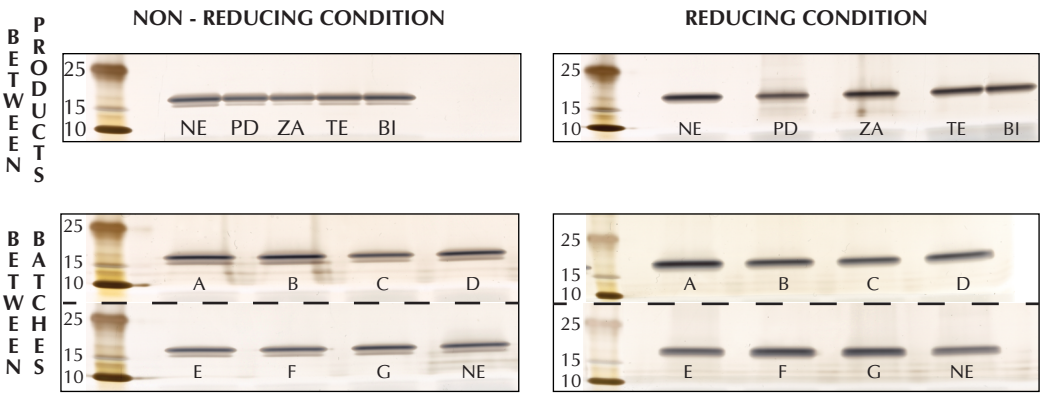

Figure 1. Filgrastim products on 4-12% Bolt® BisTris gel visualized by silver staining. Neupogen (NE), PDgrastim (PD), Zarzio (ZA), Tevagrastim (TE) and Biocilin (BI) as well as additional batches of Neupogen (A-G).

Similarly, HP-SEC revealed no impurities *i.e.* aggregation and fragments in nearly all products including the expired batches of Neupogen (**Fig. 2**). Only in the case of PDgrastim (inset **Fig. 2**) did we detected filgrastim dimer eluted at 16.9 min and oligomer eluted at 16.0 min. The total peak area percentage (%) representing dimer and oligomer compared to total area of all peaks in the chromatograms were found to be ~3.6% and ~0.1%, respectively. Particularly the peak area % of dimer was found to be higher than the limit *i.e.* 2% stated in the Ph. Eur. monograph for filgrastim concentrated solution[23]. Filgrastim monomer peak was eluted at 19.3 min in all samples, similar to the retention time of filgrastim stated on Ph. Eur. monograph for filgrastim concentrated solution[23]. The peak eluted at 23 min corresponds to the dilution buffer.

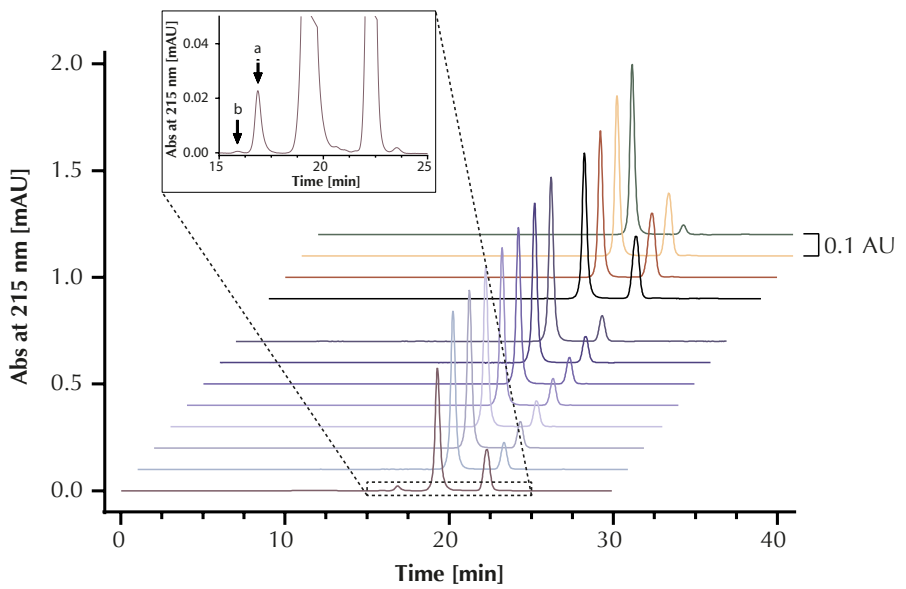

Figure 2. HP-SEC chromatograms of (top to bottom) Biocilin, Tevagrastim, Zarzio, and Neupogen followed by seven batches of expired Neupogen and PDgrastim. The inset is the zoom of PDgrastim's chromatogram. Peak (a) is filgrastim dimer and peak (b) is filgrastim oligomer.

As summarized in **Table 2**, we used HP-SEC to measure the monomer content of all filgrastim products based on standard calibration of filgrastim CRS where the peak areas of the chromatograms were plotted against the concentration of filgrastim CRS. Linearity was observed in a concentration range from 0.05 to 1 mg/mL (R^2=0.9975). Both Tevagrastim and Zarzio showed comparable filgrastim monomer content compared to Neupogen. The filgrastim monomer content of Neupogen was twofold greater than that of Biocilin. Meanwhile, the non-expired batch of Neupogen showed 1.8-fold higher filgrastim monomer content than expired batches. In contrast, the filgrastim monomer content of Neupogen was 2.4-fold higher than that of PDgrastim. The ratio between the total peak areas of filgrastim monomer detected by fluorescence and UV detectors was comparable for all tested products except PDgrastim, indicating similar structural properties.

Table 2. Comparison of Content and Potency of Tested Filgrastim Products

Trade name	Content HP-SEC, UV215 nm [mg/mL]	*In vitro* potency [Lower-Upper limit]	Specific activity [MU/mg]	total AUC FLR / total AUC UV280 nm
Neupogen	0.647 ± 0.022	24.1 MU/0.5 mL [23.1 – 25.1]	74.4	719.2 ± 6.6
Zarzio	0.650 ± 0.028	30.3 MU/0.5 mL [29.1 – 31.6]	93.2	737.6 ± 5.1
Tevagrastim	0.691 ± 0.027	31.1 MU/1.0 mL [29.8 – 32.4]	89.9	735.4 ± 6.9
Biocilin	0.317 ± 0.016	23.1 MU/1.2 mL [22.2 – 24.1]	60.7	732.4 ± 22.9
Neupogen (A)	0.360 ± 0.017	40.5 MU/1.6 mL [38.9 – 42.2]	70.3	730.9 ± 27.0
Neupogen (B)	0.353 ± 0.016	39.8 MU/1.6 mL [38.2 – 41.5]	70.4	741.6 ± 8.0
Neupogen (C)	0.358 ± 0.016	41.6 MU/1.6 mL [39.9 – 43.3]	72.6	747.0 ± 21.8
Neupogen (D)	0.354 ± 0.016	42.1 MU/1.6 mL [40.4 – 43.8]	74.3	737.4 ± 27.2
Neupogen (E)	0.353 ± 0.017	41.1 MU/1.6 mL [39.4 – 42.8]	72.7	737.3 ± 11.1
Neupogen (F)	0.359 ± 0.016	39.3 MU/1.6 mL [37.7 – 40.9]	68.4	738.1 ± 11.4
Neupogen (G)	0.368 ± 0.015	41.9 MU/1.6 mL [40.2 – 43.7]	71.2	729.4 ± 17.0
PDgrastim	0.258 ± 0.007	26.4 MU/0.5 mL [25.4 – 27.5]	102.4	669.1 ± 27.9

Identification of related proteins of filgrastim

Possible product impurity, in particular oxidized and reduced variants, contained in filgrastim products were analyzed by RP-UPLC. Native filgrastim form eluted at ~6.2 min was detected in all filgrastim products (**Fig. 3**). No oxidized forms were detected in filgrastim CRS (chromatograms not shown). In oxidative and reductive conditions, filgrastim CRS showed three oxidized peaks eluted between 5.5 min and 6.0 min and one reduced peak eluted at 6.6 min. These peaks were

detected in all batches of Neupogen, Tevagrastim and Zarzio (**Fig. 3b**). The total area percentage of either oxidized or reduced peaks was calculated for each filgrastim products and compared to the total peak area of all peaks in the chromatogram. The % peak area of each impurity *i.e.* oxidized and reduced variants for Zarzio and Tevagrastim was below the limits (1% for each impurity and 2% for total impurities) stated on Ph. Eur. monograph for filgrastim concentrated solution and were comparable to Neupogen[23].

Biocilin contained two additional peaks eluted at 5.8 min and 6.5 min (**Fig. 3b**, dotted arrows). The former peak was also apparent in the chromatogram of PDgrastim. While the % peak area of each impurity in Biocilin was below the limits, PDgrastim contained % peak area of oxidized variants *i.e.* ~2.1% higher than the acceptable limits. In contrast, expired batches of Neupogen showed comparable % peak area of oxidized and reduced variants to non-expired batch (**Fig. 3a**).

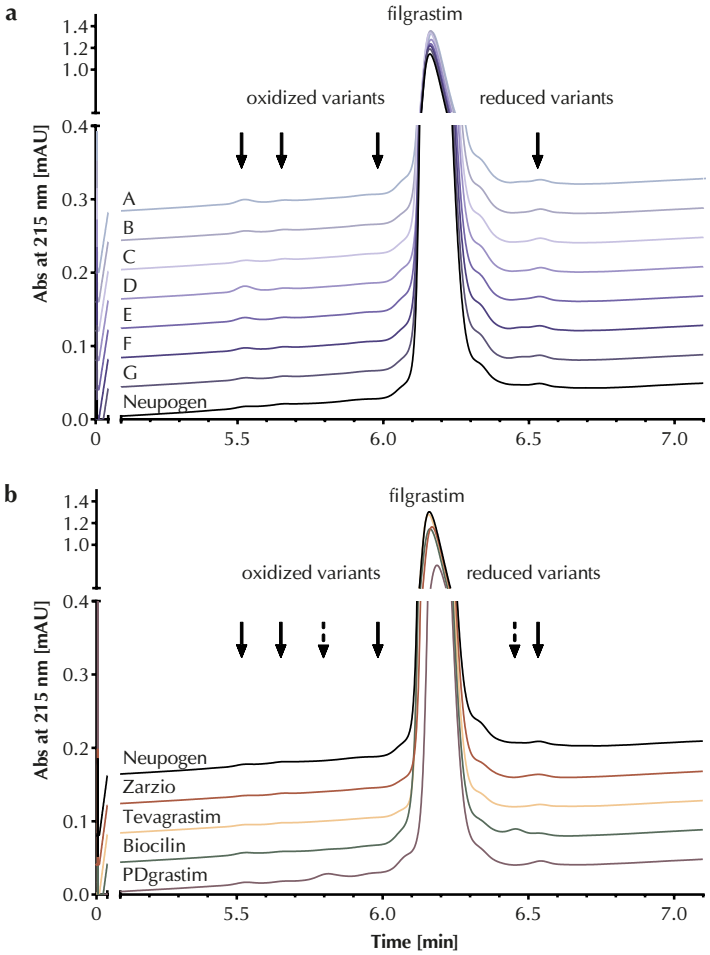

Figure 3. RP-UPLC chromatograms of (**a**) batches of Neupogen and (**b**) other filgrastim products compared to non-expired Neupogen. Dotted arrows indicate additional oxidized or reduced peaks observed only in Biocilin and/or PDgrastim.

Evaluation of bacterial endotoxin in filgrastim products

Endotoxin may be introduced during manufacturing, especially in the case of filgrastim due to its *E. coli* derivation. Hence, it is critical to quantify endotoxin in drug products because the presence of endotoxin can result in pyrogenic responses and may affect immunogenicity of the finished products. **Figure 4** shows that all filgrastim products contained an amount of endotoxin less than the limit 2 IU/mg of protein as stated in the Ph. Eur. monograph on filgrastim concentrated solution[23].

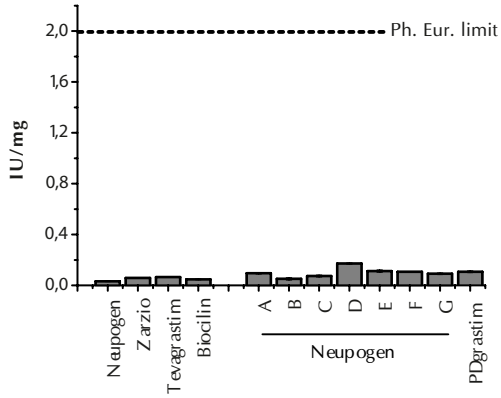

Figure 4. Endotoxin level of all filgrastim products. Seven expired batches different lots of Neupogen are represented by alphabets (A-G). Endotoxin limit as stated on the Ph. Eur. is indicated by dotted line.

Characterization of subvisible particles in filgrastim products

Subvisible particles in the range of 1-100 μm were sized and counted by MFI. As it becomes imperative to distinguish non-proteinaceous particles *i.e.* silicone oil from proteinaceous particles, image filtering capabilities of MFI, namely aspect ratio and intensity minimum were used as described elsewhere[24]. **Figure 5** exhibits representative images of proteinaceous particles and silicone oil droplets. In contrast to the uniform circularity of the silicone oil droplets, the proteinaceous particles are highly heterogeneous in shape and size, ranging from small translucent ovals of 5 μm, protofibers to oligomers around 40 μm.

Subsequently, we quantified the particle concentrations as listed in **Table 3**. Neupogen supplied in PFS had much higher concentration of both proteinaceous particle and silicone oil droplet than other analyzed products. Tevagrastim, although also supplied in PFS, contained less subvisible particles than Neupogen. Filgrastim products that are available in vials, including Biocilin and expired batches of Neupogen (A, B, E and F) had less than 10,000 particles/mL of silicone oil droplets. In all cases, the average number of particles presents in the analyzed filgrastim products was below the limit of 3,000 particles ≥ 10 μm/container and 300 particles ≥ 25 μm/container as stated in the Ph. Eur. monograph for particulate contamination: sub-visible particles[26].

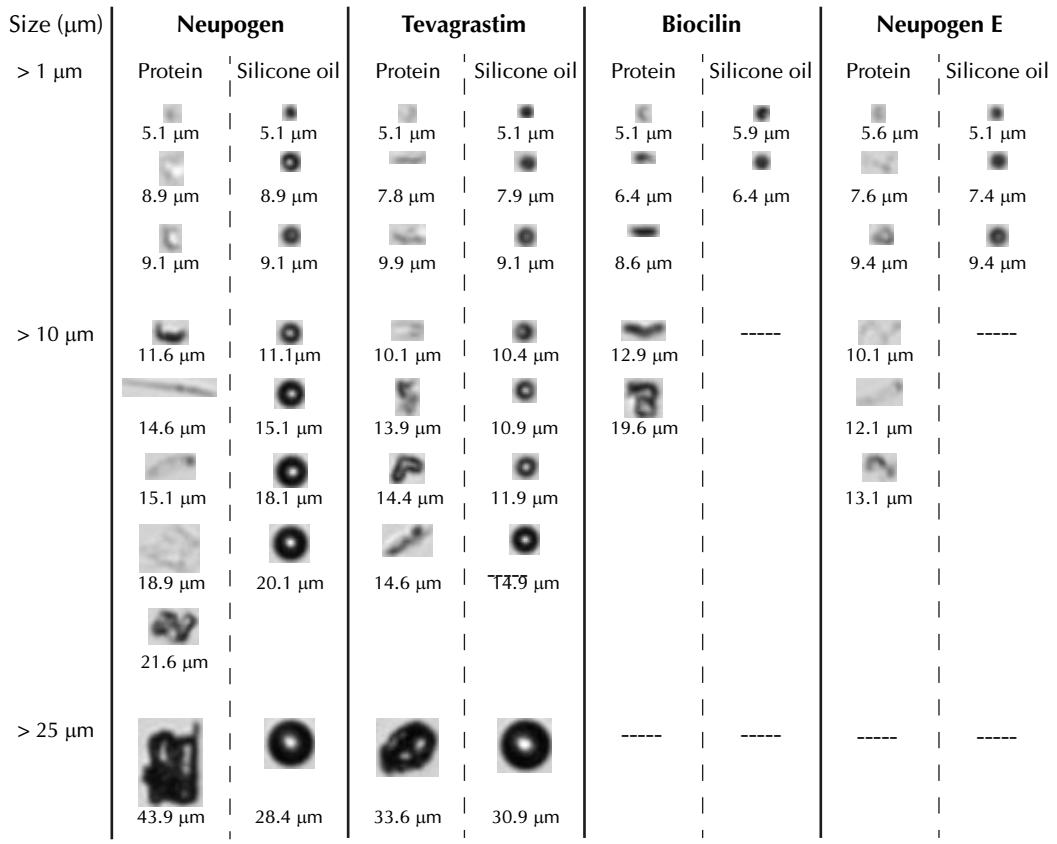

Figure 5. Representative images of proteinaceous particles and silicone oil droplets observed by MFI in selected filgrastim products.

Determination of *in vitro* potency of filgrastim products

The *in vitro* potency of filgrastim products was measured by comparing their proliferative effect in M-NFS-60 cells with 2nd WHO G-CSF IS. **Table 2** lists the estimated potencies of all products. *In vitro* potencies of most products were between 80-125% of the declared potency. These meet the specification as set in the Ph. Eur. monograph for filgrastim concentrated solution[23], with the exception of Biocilin, for which there is no stated potency. Specific activity of all products analyzed was determined based on the filgrastim monomer content. All expired batches of Neupogen had comparable specific activity to a non-expired batch. While Tevagrastim and Zarzio showed slightly higher specific activity than Neupogen, specific activity of PDgrastim was substantially higher. In contrast, Biocilin exhibited lower specific activity than Neupogen.

Table 3. Comparison of Proteinaceous Particles and Silicone Oil Droplets Concentrations (particles/mL) in Some Filgrastim Products Measured by MFI

Trade Name	Size Range					
	Proteinaceous particles			Silicone oil droplets		
	> 1 μm	> 10 μm	> 25 μm	> 1 μm	> 10 μm	> 25 μm
Neupogen	79,411	57	4	47,918	241	8
Tevagrastim	31,959	31	4	10,987	38	4
Biocilin	8,591	8	0	2,602	0	0
Neupogen (A)	10,265	4	4	4,555	0	0
Neupogen (B)	17,713	50	0	9,997	8	0
Neupogen (E)	21,080	15	0	8,461	0	0
Neupogen (F)	22,126	11	8	7,666	19	4

DISCUSSION

The current study showed that the analyzed filgrastim products approved by the EMA (Zarzio and Tevagrastim) were comparable to the innovator product (Neupogen), whereas the copy filgrastim products (Biocilin and PDgrastim) differ substantially in specific activity.

While the stated concentration of Biocilin (0.25 mg/mL) is 2.4-fold lower than Neupogen (0.6 mg/mL), we calculated a higher filgrastim monomer content of Biocilin, as is evident from by HP-SEC. At the same time, Biocilin showed lower specific activity compared to Neupogen. The lower specific activity may indicate the presence of impurities or denatured protein. Biocilin was licensed in Mexico before the biosimilar guidelines were put in place, which may explain why the stated potency of Biocilin is lacking[27]. Nonetheless, recent pharmacovigilance study conducted in Mexico for filgrastim copy products including Biocilin in cancer patients detected no new adverse events, suggesting that observed quality differences in Biocilin seem to have no impact on product safety[28]. This issue deserves further study.

PDgrastim and several batches of Neupogen were received close to their expiration dates. Even so, to give an insight into the quality of expired copy filgrastim product compared to the innovator, we included them in our analysis as worst case scenarios. Only the expired batches of PDgrastim showed noticeable deterioration in quality. The filgrastim monomer content of Neupogen was 2.4-fold higher than of PDgrastim while declared content showed a twofold difference. Albeit with lower content, PDgrastim exhibited substantially higher specific activity compared to Neupogen, which could be due to differences in structure as demonstrated by HP-SEC. Other factors which may account for differences in specific activity include different standards or assays used. In addition to specific activity, PDgrastim also showed higher % area of dimer and of oxidized variants. No differences were observed in any expired batches of Neupogen.

We analyzed two copy filgrastim products, but it should be considered that more products are available in less regulated markets. Testing these products will provide more insight in the quality of available copy filgrastim products in general. Additionally, future studies should include more assays to provide more robust comparative quality data.

CONCLUSION

Here, we report the head-to-head comparative quality study of biosimilar and copy filgrastim products. Using selected analytical tools and *in vitro* bioassay, we could demonstrate no clinical significant differences in quality between the filgrastim products studied except for the specific activity of the two filgrastim copy products. Looking forwards, regulators and manufacturers should put more effort into making such comparative quality studies.

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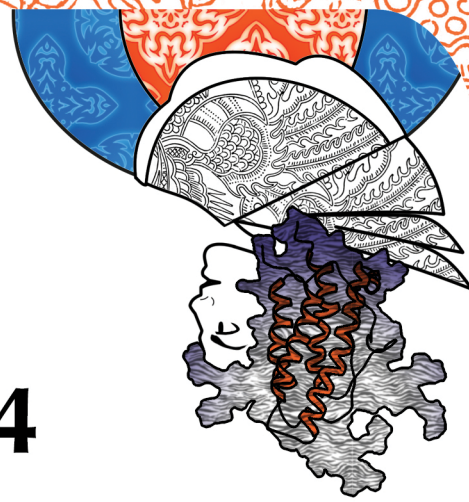

Chapter 4

Quality and Batch-to-Batch Consistency of Original and Biosimilar Epoetin Products

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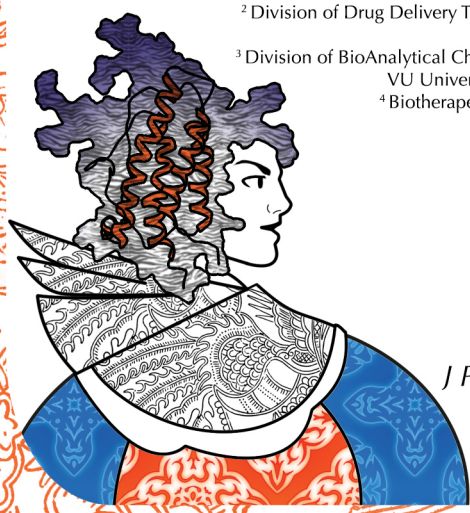

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ABSTRACT

Comprehensive physicochemical characterization and biological assays are essential parts in assessing quality attributes of biologicals. Here, we compared the quality of different marketed recombinant human erythropoietin (epoetin) products: originators, Eprex and NeoRecormon as well as 2 biosimilars, Retacrit and Binocrit. In addition, assessment of batch-to-batch variability was included by collecting 2 or more batches of each product. Common assays which included sodium dodecyl sulfate-polyacrylamide gel electrophoresis, high–performance size-exclusion chromatography, asymmetrical flow field-flow fractionation, capillary zone electrophoresis and potency testing were used. Of the tested products and among batches of single products, variations in epoetin content, isoform profiles, and potency were found. Ultimately, this study demonstrated the high quality of epoetin products with some degree of variation among products and batches, confirming the "similar but not identical" paradigm of biologicals.

KEYWORDS recombinant human erythropoietin, biosimilar, immunogenicity, physicochemical properties, protein characterization

INTRODUCTION

Since the 1980s, the advent of recombinant DNA technology has enabled the development of many innovative recombinant human therapeutic proteins.[1,2] These products have enabled the treatment of a variety of diseases and have become the fastest growing class of therapeutics. Recombinant human erythropoietin (epoetin) was one of the first authorized recombinant proteins on the market. It is mainly used for the treatment of anemia in patients with chronic kidney disease and cancer.[3,4]

Severe side effects, such as thromboembolic processes and antibody-associated pure red cell aplasia (PRCA) are rare. PRCA may occur if epoetin-induced antibodies are able to neutralize the native endogenous erythropoietin.[5,6] Epoetin shares its factors for immunogenicity with nearly all therapeutic proteins. The exact mechanisms underlying immunogenicity are still not fully understood. Multiple factors including product-related factors (formulation, contaminants, glycosylation and impurities), storage and handling, route of administration, and patient characteristics play a role in this.[7,8]

Since 2006 the loss of patent and data protection has allowed the introduction of generic versions of therapeutic proteins such as somatropin, filgrastim, and epoetin. However, the generic regulatory route used for small molecules cannot be used for proteins. Owing to their inherent variability, complexity, and heterogeneity, it is impossible to establish that 2 protein products are identical[9,10]. Individual protein products themselves also demonstrate micro-heterogeneity and batch-to-batch variability so cannot be identical to themselves. Therefore, regulatory frameworks have been established throughout the world requiring an extensive comparison in quality, efficacy, and safety to show similarity between the original product and the intended copy.[11,12] If the criteria are met, the duplicate product can be marketed as a biosimilar.

As we had access to 4 marketed epoetin products, 2 originators, Eprex and NeoRecormon and two biosimilars, Retacrit and Binocrit, we performed quality assessment for these products. Eprex (epoetin alfa) and NeoRecormon (epoetin beta) have been reported to differ in their isoform compositions and biological properties on account of the use of different CHO cells strain.[13]both synthesized in Chinese hamster ovary cells, which are used for the treatment of erythropoietin (EPO). Meanwhile, the quality assessment of Retacrit and Binocrit to their reference product, Eprex, has been shown elsewhere to have slight variation in their quality attributes.[14,15]

Besides quality, batch consistency is also considered important for biologicals. Although a few studies have looked into batch-to-batch variability of an individual epoetin brand,[13,16,17] there has been no published study on batch-to-batch consistency of multiple epoetin brands marketed in Europe. As we also had the possibility to collect multiple batches from these 4 epoetin products, this comparability study is feasible as a follow-up to a study we published earlier.[14]

MATERIALS AND METHODS

Epoetin products

All epoetin products (see **Table 1** for an overview) were either obtained from local pharmacies in the Netherlands or provided by Hospira and Sandoz. They were received in the original prefilled syringes and stored as stated on the product specification. As an internal reference standard, epoetin-biological reference preparation (BRP) batch 3 (EDQM, Strasbourg, France) was included in every experiment to validate the method as recommended in the European Pharmacopeia (Ph. Eur.) monograph on Erythropoietin concentrated solution.[18] It contains equals parts of epoetin alfa and beta.[19] Before every test, visual inspection was performed for the potential presence of visible particles. All products remained clear and colorless. In all cases, products were tested within their shelf lives.

Table 1. List of All Epoetin Products

Brand Name (INN)	Lot Number	Declared Potency	Excipients
Eprex (epoetin alfa)	DDS5L00 DGS4W00 DHS5T00 DIS3M00	4,000 IU/0.4 ml	Sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, sodium chloride, glycine, polysorbate 80
Binocrit (epoetin alfa)	341211 450112 730412	8,000 IU/0.8 ml 10,000 IU/1.0 ml	Sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, sodium chloride, glycine, polysorbate 80
Retacrit (epoetin alfa)	8K058L8 8M072C9 9F081G9 9M108N9	10,000 IU/1.0 ml	Disodium phosphate dihydrate, sodium dihydrogen phosphate dihydrate, sodium chloride, calcium chloride dihydrate, polysorbate 20, glycine, leucine, isoleucine, threonine, glutamic acid, phenylalanine
NeoRecormon (epoetin beta)	H0002H01 H0003H01	30,000 IU/0.6 ml	Urea, sodium chloride, polysorbate 20, sodium dihydrogen phosphate dihydrate, disodium phosphate dodecahydrate, calcium chloride dihydrate, glycine, l-leucine, l-isoleucine, l-threonine, l-glutamic acid, l-phenylalanine

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

The epoetin products were loaded on 5% polyacrylamide gel (stacking section) and separated on 15% polyacrylamide gel (running section) under nonreducing conditions as previously described by Brinks *et al.*[14] Unless indicated otherwise, all materials were obtained from Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands). In short, loading solutions of all epoetin products included 24 µL of undiluted products and 6 µL of 5x sample buffer (containing Tris-HCl pH 6.8, glycerol, sodium dodecyl sulfate and bromophenol blue). Two micrograms of epoetin- BRP batch 3 were included on each gel.

Before loading, all samples were incubated either at 95°C, 70°C, or room temperature (25°C) for 10 min to facilitate protein unfolding. PageRuler™ Prestained Protein Ladder, 10-180 kDa (Life Technologies, Bleiswijk, the Netherlands) was used as reference for molecular weight in all cases. Separation was performed on Mini-PROTEAN® II Electrophoresis Cell with the following running conditions: 30 min at 70 V, followed by 60 min at 150 V. Protein bands were visualized by silver staining method as described by Brinks *et al.*[14]

High–Performance Size–Exclusion Chromatography

During the course of this study, the collection of multiple batches of each epoetin product was rather difficult. Hence, epoetin products were obtained at different time points. Retacrit and NeoRecormon were obtained back in 2010. Subsequently, Binocrit and Eprex were obtained in the early and late 2014, respectively. As there was an urge to analyze unexpired products, high–performance size–exclusion chromatography (HP–SEC) was first performed on a Waters 2695 Separations Module connected to a Waters 2487 Dual λ Absorbance Detector (Waters Corporation, Milford, MA) for the first 2 products. The machine was then no longer available and we had to switch to an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) combined with a Wyatt Eclipse (Wyatt Technology Europe GmbH, Dernbach, Germany) to analyze the later products.

On both machines, a Tricorn™ high-performance Superdex 200 10/300 GL column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was installed. Auto sampler (Agilent) temperature was set at 4°C, and each time, 100 µL of undiluted product were injected. The eluent was 14.4 g/L $Na_2HPO_4.2H_2O$ (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.2 g/L KH_2PO_4 and 23.4 g/L NaCl (Merck, Darmstadt, Germany) at pH 7.4 and filtered through a 0.2-µm filter (Sartorius Stedim, Göttingen, Germany).

Separation took place at a flow rate of 0.5 mL/min for 60 min at 30°C. Absorbance was recorded at 280 nm and analyzed using either Empower 2 software version 6.20.00.00 or Astra software version 5.3.4.20. A DAWN® HELEOS™ 18–angle laser light scattering (MALLS) was part of the Agilent system, therefore allowing estimation of the average molecular weight of eluting compounds. Alternatively, proteins with different molecular weights, (1) lysozyme, (2) trypsin, (3) ovalbumin, (4) albumin and (5) holo-transferrin, were used on the Waters system as calibration standards for molecular weight estimation. All proteins were purchased from Sigma-Aldrich.

Subsequently, the protein content was determined from the UV signal at 280 nm using Beer-Lambert law. For all epoetins, a molar extinction coefficient of 22,600 M^{-1} cm^{-1} was used.[20] One-IU epoetin was set to 8.4 and 8.3 ng epoetin protein for epoetin alfa[21,22]/zeta[23] and beta[24] ,respectively.

Asymmetrical Flow Field–Flow Fractionation

Asymmetrical flow field-flow fractionation (AF4) was performed on an Agilent 1200 HPLC system (Agilent Technologies) with degasser, cooled auto sampler, and a UV (280 nm) and a fluorescence detector. It was combined with a Wyatt Eclipse (Wyatt Technology Europe GmbH, Dernbach, Germany) and a DAWN® HELEOS™ 18-angle laser light scattering (MALLS) detector (Wyatt Technology Europe GmbH). Fifty microliters of each undiluted formulation were injected through

a 350-µm thick, medium wide-spaced in a small channel with a Nadir 5-kDa cutoff regenerated cellulose membrane (Wyatt Technology Europe GmbH). The same mobile phase buffer was used as in HP–SEC but was filtered through a 0.1-µm cellulose nitrate Whatman™ filter (GE Healthcare Life Sciences, Pittsburgh, KS). The detector flow and the focus flow were set to 1 and 1.5 mL/min, respectively. The Eclipse elution settings are summarized in **Table 2**. Calculation of the molecular weight from the MALLS and UV signals was performed by the Astra software version 5.3.4.20. Protein content was determined as described before.

Table 2. AF4 Elution Program Settings

	Start (min)	Duration (min)	Crossflow (mL/min)
Elution	0	2	1.8
Focus	2	1	1.5
Focus + Injection	3	2	1.5
Elution	5	10	1.8
Elution	15	10	0

Enzyme–Linked Immunosorbent Assay

Epoetin was identified with the Quantikine IVD Human Epoetin ELISA (R&D Systems Europe, Abingdon, Oxon, United Kingdom) according to the manufacturer's instructions. After fractionation by HP–SEC as described by Hermeling *et al.*,[25] 20 µL of each fraction was added to a well containing 100 µL of assay diluent buffer and 80 µL of specimen diluent buffer, provided by the kit. Chromogen was left to react for 20 min before it was stopped by the addition of acid. The plate was immediately read on an Infinite® M1000 PRO microplate reader (Tecan, Giessen, the Netherlands) at 450 nm and 600 nm (reference wavelength). In each plate, both recombinant human epoetin provided in the kit and Eprex were used as the standards.

Capillary Zone Electrophoresis

The isoform distribution of different epoetin products was assessed by capillary zone electrophoresis (CZE) according to the Ph. Eur. monograph on Erythropoietin concentrated solution.[18] Binocrit was analyzed on a 7100 CE System equipped with a photodiode array detector and ChemStation software from Agilent Technologies (Wilmington, DE); the remaining epoetin products were analyzed on a ProteomeLab™ PA800 or a PA800 Plus Pharmaceutical Analysis System coupled to UV detector and operated with 32 Karat software from Beckman Coulter (Brea, CA). Epoetin internal reference standard (Epoetin-BRP batch 3) and products were pretreated by direct loading to either Nanosep® (Pall Corporation, Ann Arbor, MI) or Amicon® Ultra (Sigma-Aldrich) centrifugal devices, both with a molecular weight cutoff value of 10 kDa as described in the Ph. Eur. monograph. Retentates were aliquoted and stored at –80°C until just before separation.

An uncoated 50 µm inner diameter fused-silica capillary with an effective length of 100 cm (Polymicro Technologies, Phoenix, AZ) was used for separation. The CZE buffer consisted of 0.01–M tricine (Acros Organics, Geel, Belgium), 0.01–M sodium chloride (Merck), 0.01–M sodium acetate (Merck), 7–M urea (Amresco, Solon, OH) and 25–mM putrescine (Sigma-Aldrich), pH 5.55

adjusted with 50% (v/v) glacial acetic acid at 30°C and filtered through Minisart® 0.45–µm filter (Sartorius Stedim). The preconditioning of the capillary and between-run rinsing was performed adapting either Ph. Eur. monograph or Zhang *et al*[26]. In both methods, epoetin internal reference standard and products were injected hydrodynamically at 0.7 psi for 40 s with a separation voltage of 143 V/cm. The UV detector set 214 nm was operated at 2 Hz. The isoform distribution was assessed from 3 or more independent runs of every batch.

In Vitro Bioassay

An *in vitro* bioassay was performed using the erythropoietin-dependent subline, UT-7/EPO derived from a human erythroleukaemia.[27] Cells were maintained in Iscove's modified Dulbecco's medium containing 10% heat-inactivated fetal calf serum supplemented with L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (0.05 mg/mL) and 0.2 IU/mL epoetin. Cells were sub-cultured every 2-3 days and split 1:5 when they had reached a cell density of 2-5 x 10^5 cells/mL.

Two-fold dilutions of the epoetin samples ranging from 0.1 IU/mL to 0.00078 IU/ml were incubated with UT-7/EPO cells at a density of 0.5 x 10^4 cells/well. The plates were incubated at 37°C, 5% CO_2 for 48 h and 3H–thymidine (thymidine[methyl–3H] 1 mCi (37 MBq])/mL, Perkin Elmer, Beaconsfield, United Kingdom) 0.5 µCi/well, diluted in assay medium, added for the last 4 h of the incubation period. The cells were harvested onto glass fiber filter mats using a micro 96 harvester (Molecular Devices, Wokingham, United Kingdom) and the radioactivity incorporated into DNA estimated by scintillation counting using a 2450 MicroBeta2 scintillation counter (Perkin Elmer). Bioactivity estimates of the different preparations were derived relative to the epoetin standard (Third WHO International Standard [third WHO IS] for erythropoietin, recombinant, for bioassay, 11/170 available from NIBSC, United Kingdom).

In Vivo Potency Test

On account of ethical considerations in the use of animals, only selected epoetin products and batches were assessed for potency in normocythemic mice by measuring the stimulation of reticulocyte production according to the Ph. Eur. for erythropoietin concentrated solution.[18] BALB/c female mice received the third WHO IS for erythropoietin and the epoetin products, diluted into a high, middle and low dose, subcutaneously. Each dilution group consisted of 6 animals weighing between 16 and 23 g. Mice were kept for 5 days, and blood was withdrawn from the orbital sinus before culling by cervical dislocation. At the end of the assay, reticulocyte concentration as a percentage of total erythrocyte concentration was determined. Potency estimates for the epoetin products were calculated relative to the epoetin standard, by fitting a parallel-line model comparing assay response to log concentration using CombiStats version 5.0 (1999-2013 EDQM/Council of Europe). Assay validity was assessed by analysis of variance with non-linearity and nonparallelism considered significant at the 1% level ($p < 0.01$). Duplicate potency estimates from independent bioassays were combined using CombiStats version 5.0.

RESULTS

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Possible proteinaceous impurities in the products were checked with sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. In all tested products, a single broad band of epoetin was apparent on silver staining (**Fig. 1**), corresponding in position and intensity to the single band of the epoetin-BRP batch 3. Neither higher molecular weight species nor fragments were found in any batch. In addition, different sample preparations, that is, incubation at either 95°C, 70°C or room temperature (RT) did not induce aggregation and/or degradation. Faint bands identified in the Retacrit and NeoRecormon samples at 43 kDa and higher were likely due to overloading of prestained protein marker.

High–Performance Size–Exclusion Chromatography

High–performance size–exclusion chromatography (HP–SEC) was used to characterize soluble aggregates and to quantify epoetin. As demonstrated in **Figure 2**, the main epoetin peak of Eprex and Binocrit (panels a and b) separated on an Agilent HPLC system was detected at 29.9 and 30.1 min, respectively. The monomer identities of both epoetin alfa products showed comparable average molecular weight close to the theoretical value of 30.4 kDa, as estimated by MALLS (**Supplementary Fig. 1**, filled bars).[28] The additional peaks before the main epoetin peak are probably related to the use of polysorbate 80 (PS80) as a stabilizer. This finding has also been reported by Hermeling *et al.*[25] and will be discussed further below.

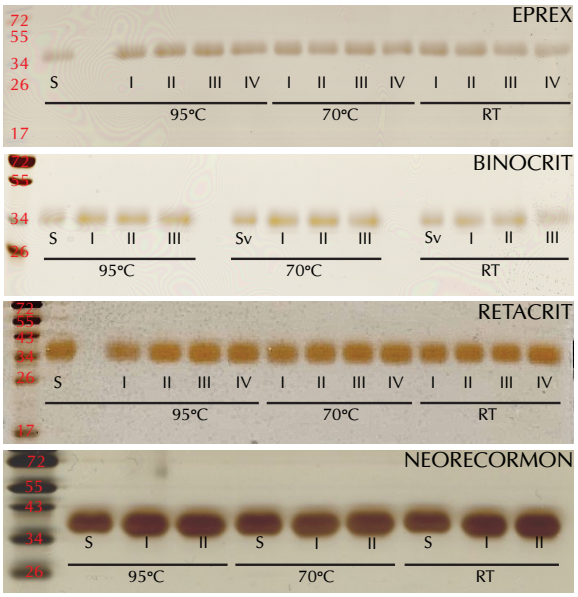

Figure 1. SDS–PAGE of all epoetin products under nonreducing condition. Roman number represents the different batches of each products. S is epoetin-BRP, and RT stands for room temperature. Different intensities are most likely due to gel-to-gel variation.

On a Waters system, the main epoetin peak eluted at 29.3 and 28.2 min for Retacrit and NeoRecormon, respectively (**Figs. 2b** and **2c**). Although Retacrit eluted about 1 min later, its average molecular weight was found to be similar to that of NeoRecormon, as estimated by the calibration standards of protein with known molecular weight (**Supplementary Fig. 1**, empty bars). Here, 3 possible explanations are suggested. First, the method variability of using proteins with known molecular weight to estimate the molecular weight of epoetin might be a factor. Hence, difference of 1 min in elution time cannot be precisely measured in terms of molecular weight. It may also explain the larger estimated average molecular weight of epoetin monomer (57.1 kDa) than the theoretical value (30.4 kDa). Second, the difference in elution time but similar estimated molecular weight suggests possible different hydrophilic interactions between the column materials and the 2 types of epoetin. Third, differences in glycosylation pattern may lead to differences in the amount of bound water and hence the hydrodynamic volume of the proteins.

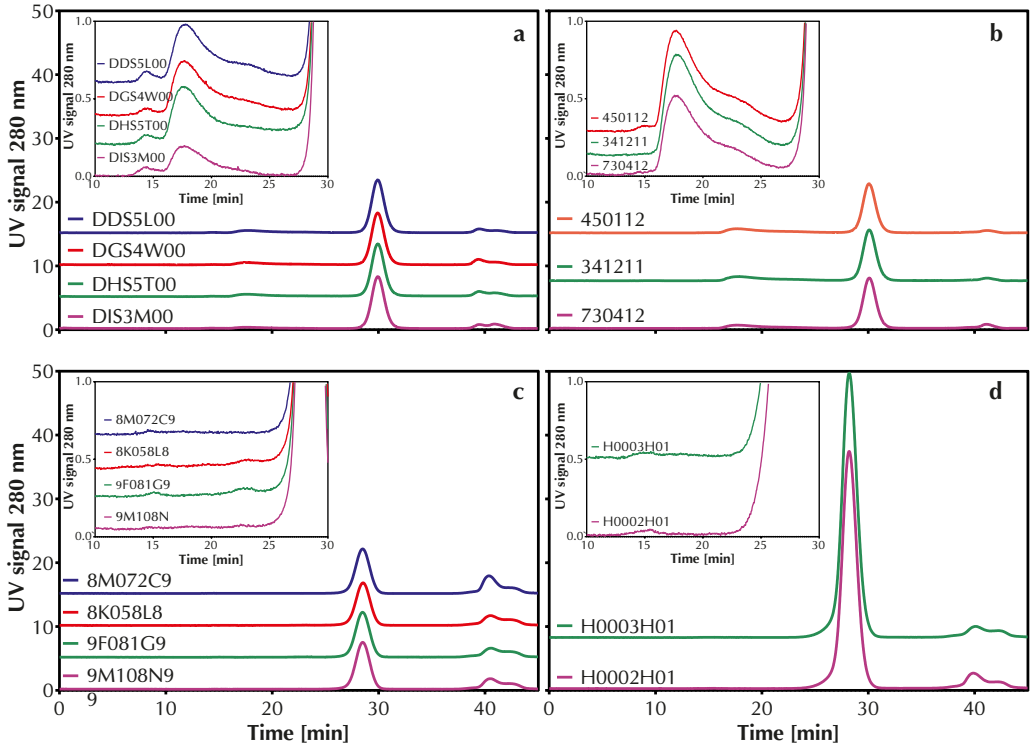

Figure 2. HP–SEC chromatograms of (**a**) Eprex, (**b**) Binocrit, (**c**) Retacrit and (**d**) NeoRecormon. The inset is a zoom of the chromatograms of the area between 15 and 30 min. The different colors represent the batches of single product.

To study whether the high–molecular weight (HMW) peak was due to PS80 or epoetin oligomers or both, (1) 0.3 mg/mL PS80, (2) Eprex, and (3) Eprex spiked with 0.3 mg/mL PS80 were applied onto the column. As shown in **Figure 3a**, the retention times of PS80 (peak 1) and Eprex HMW (peak 2) partly overlap. When spiking Eprex with 0.3 mg/mL PS80, the shoulder of Eprex HMW increased significantly and the HMW peak itself shifted to a slightly longer retention time (compare peak 2 and peak 3). Conversely, the PS80 peak was slightly shifted in the presence

of epoetin (compare peak 1 and peak 3). These results indicate that epoetin affects the elution behavior of PS80, *vice versa*, which compromises an accurate assessment of aggregate content.

In an attempt to overcome this, we added an equal concentration of PS80 to the mobile phase buffer as present in Eprex (0.3 mg/mL), with the intention to avoid any PS80 signals.[25] However, although the concept worked for placebo formulation, and for the Eprex formulation, the peak became smaller, and for the latter sample, a negative peak appeared (**Fig. 3b**), again indicating that epoetin and PS80 mutually influence each other's elution behavior.

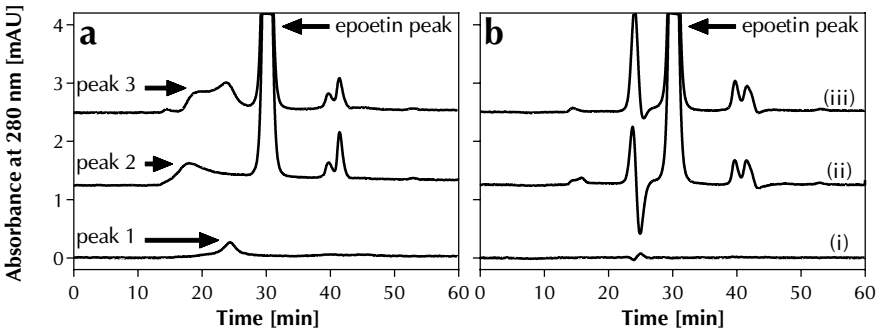

Figure 3. Zoomed HP–SEC chromatograms of (i) PS80, (ii) Eprex and (iii) Eprex spiked with 0.3 mg/mL PS80 in (**a**) absence or (**b**) presence of 0.3 mg/mL PS80 in the mobile phase.

These data show that it is impossible to accurately determine the amount of epoetin within the HMW species peak. Therefore, as adapted from Hermeling *et al.,*[25] fractions (250 µL) of Eprex (DDSL500) were collected from 10 min until 35 min, and the epoetin content therein was assessed by enzyme-linked immunosorbent assay. As shown in **Figure 4**, there was no detectable epoetin dimer or oligomer in the region where HMW species eluted (15-25 min). This implies that the different elution behavior of PS80 alone, PS80 in Eprex formulation, and Eprex spiked with PS80 was not solely influenced by epoetin. Instead, buffer components or denatured protein could also affect the influence behavior of PS80.[29] Furthermore, the detected HMW species might as well consist of denatured protein which was not detected by the antibodies.

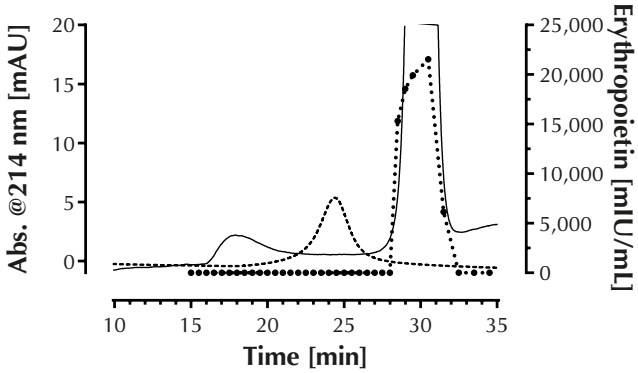

Figure 4. Chromatograms of Eprex batch DIS3M00 (solid line) and 0.3 mg/mL of PS80 (dashed line) on Superdex 200 column (left y-axis) and results from epoetin specific enzyme-linked immunosorbent assay (dotted line) on Eprex column fractions (right y-axis).

The determination of epoetin monomer content in all batches is summarized in **Table 3**. As expected, a higher EPO monomer content was found in NeoRecormon than that in the other epoetin products, in line with the higher potency as declared on the label. The epoetin monomer content of Binocrit and Retacrit was ~3% and ~14%, respectively, less than that of Eprex. Content discrepancies between batches of 1 brand were also apparent. In Eprex, batch DDSL500 contained 2% more monomeric epoetin than in DGS4W00. Batch-to-batch variation was found to be the highest in NeoRecormon (~12%).

Table 3. Comparison of Content of the 4 Epoetin Products Tested

Brand Name (INN)	Lot Number	Declared Content [IU/ml]	Content UV280nm [IU/ml]				
			HP–SEC			AF4	
Eprex (epoetin alfa)	DDS5L00	10,000	9963	±	2	9,747	± 38
	DGS4W00		9770	±	35	9,480	± 189
	DHS5T00		9875	±	120	9,614	± 76
	DIS3M00		9825	±	100	9,587	± 38
	Mean		*9858*	±	*80*	*9,607*	± *105*
Binocrit (epoetin alfa)	341211	10,000	9803	±	34	10,817	± 265
	450112		9400	±	N.D.	10,185	± 591
	730412		9553	±	*7*	11,008	± 742
	Mean		*9623*	±	*24*	*10,670*	± *568*
Retacrit (epoetin alfa)	8K058L8	10,000	8014	±	63	9,275	± 636
	8M072C9		8496	±	52	10,059	± 700
	9F081G9		8480	±	81	9,468	± 369
	9M108N9		8808	±	44	10,450	± 587
	Mean		*8450*	±	*62*	*9,812*	± *586*
NeoRecormon (epoetin beta)	H0002H01	50,000	47,784	±	358	52,158	± 139
	H0003H01		53,841	±	92	58,582	± 569
	Mean		*50,813*	±	*261*	*55,370*	± *414*

N.D., not determined

Asymmetric Flow Field–Flow Fractionation

Asymmetric flow field–flow fractionation (AF4) was used as an orthogonal method to HP–SEC for the separation and quantification of various sizes of protein monomer and aggregates. As shown in **Figure 5**, 2 or more distinct peaks were detected in all epoetin products. The elution time of epoetin monomer slightly differed between products. Monomers of Eprex and Binocrit, which are epoetin alfa, were detected at between 8.6 and 8.8 min. Monomers of Retacrit (epoetin zeta) and NeoRecormon (epoetin beta) were eluted slightly later at ~8.9-9.2 min. As in HP–SEC, differences in elution time between epoetin products were not reflected in the average molecular weight as estimated by MALLS (**Supplementary Fig. 1**). It shows that epoetin possibly interacts with the cellulose membrane.[30]

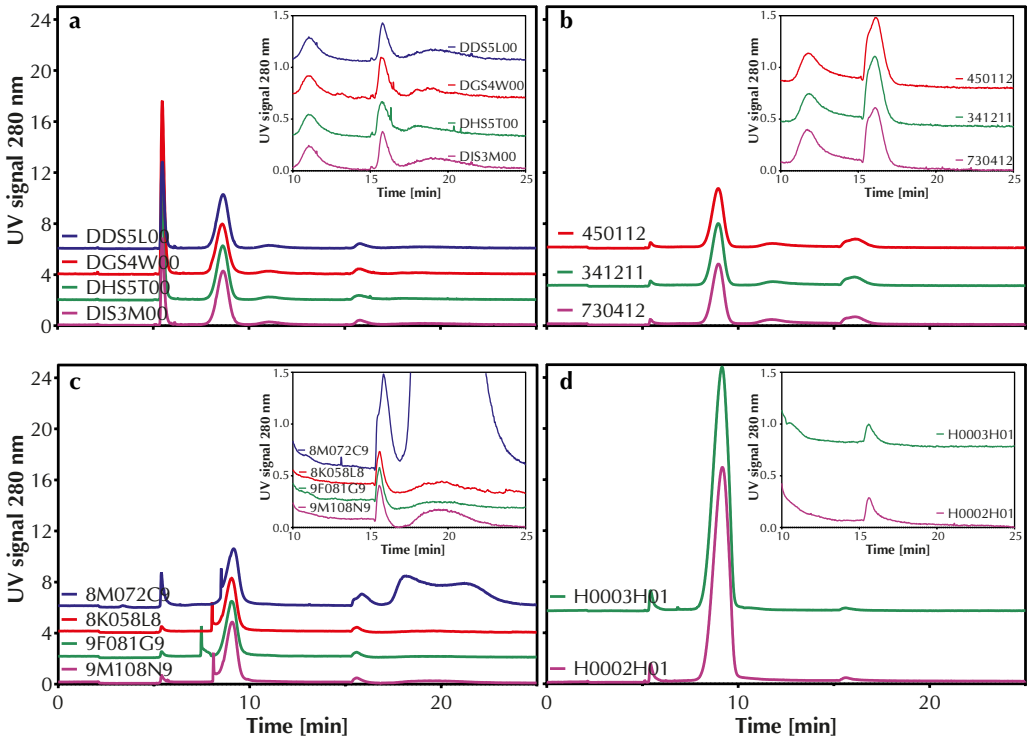

Figure 5. AF4 elugrams of the 4 epoetin products (**a**) Eprex, (**b**) Binocrit, (**c**) Retacrit and (**d**) NeoRecormon. The inset is a zoom into the elugrams in the area between 10 and 25 min. The different colors represent the batches of single product.

The peak eluting at ~11 min in both epoetin alfa products (**Figs. 5a** and **5b**) is likely related to PS80. As shown in **Supplementary Figure 2**, the peak of PS80 alone also has the same elution time. For Retacrit and NeoRecormon (**Figs. 5c** and **5d**), which contain PS20 instead of PS80, this particular peak was absent. Peaks eluting earlier than epoetin monomer (<8 min) are likely due to the excipients (listed in **Table 1**). The peaks eluting when the crossflow was stopped (>15 min) may be due to larger impurities. However, because small peaks with similar retention times were also observed when injecting placebo formulations (results not shown), it is also possible that they may result, at least in part, from contamination of the AF4 channel and tubings.

In line with the results obtained with HP–SEC, the highest epoetin monomer content was found in NeoRecormon. Also, the highest content differences between batches about ~13% were found in NeoRecormon. The variation between Eprex batches (~3%) was lower than that of other tested products. In contrast, Eprex contained the least epoetin monomer content among all epoetin products. These results clearly indicate variation in content determination between HP–SEC and AF4 most likely due to different adsorption which hinder the full mass recovery of the injected protein[30].

Capillary Zone Electrophoresis

Multiple isoforms of epoetin were detected on separation by capillary zone electrophoresis by an adapted Ph. Eur. method. Owing to the use of different instrumentation, some migration time differences were observed. To correct for this, the time scale of the electropherograms was converted to effective mobility. This conversion enabled good inter- and intraproduct comparison. In all products the effective mobility of isoforms was observed between 0.001 and 0.003 $cm^2 V^{-1} min^{-1}$ (**Fig. 6**) indicating reproducible migration behavior. Eprex and Binocrit (epoetin alfa) consisted of 6 isoforms, whereas Retacrit (epoetin zeta) contained an additional isoform. NeoRecormon, which is an epoetin beta, contained 8 isoforms, similar to the number of isoforms contained in epoetin-BRP batch 3 (data not shown). This was expected because epoetin-BRP is an equal mixture of epoetin alfa and beta.[19]

Detected peaks were annotated, and the area under the curve of each isoform in each tested batch per product was quantified as percentage of the total area under the curve. As shown in **Figure 7a**, no major variation was observed between individual batches of Eprex, implying high batch-to-batch consistency. A similar batch-to-batch consistency was also found for the other tested products (data not shown).

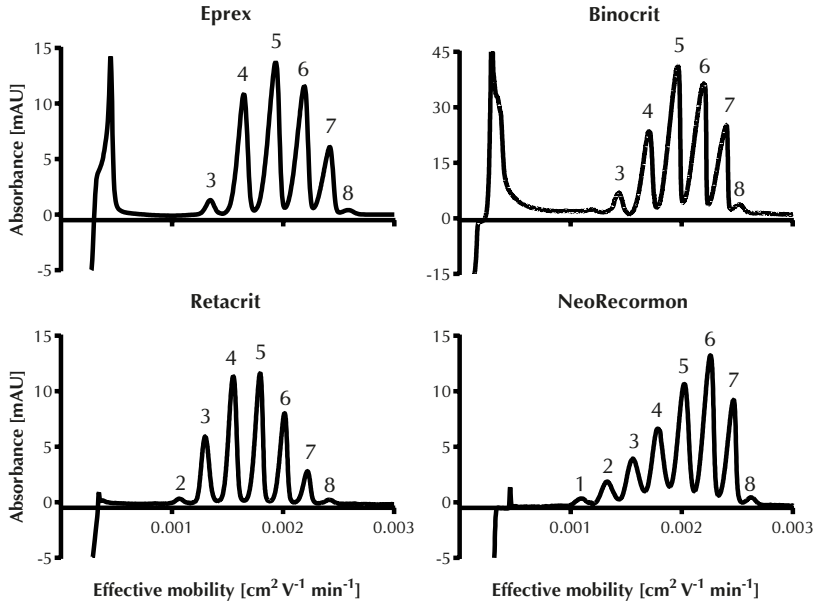

Figure 6. Representative CE-UV analysis of the 4epoetin products. Each product is represented by 1 repetition of 1 batch.

Subsequently, the average relative amount of each isoform from different products was calculated (**Fig. 7b**). Eprex and Binocrit showed a high degree of similarity, with peaks due to isoform 5+6 being the most abundant ones. In agreement with our previous study, the peak correlating to isoform 4+5 are the highest relative isoform content in Retacrit.[14] Lastly, the peak due to isoform 6 was the most abundant one in NeoRecormon. The data confirm that the relative isoform contents of tested products are within the acceptance criteria stated in the Ph. Eur. monograph on Erythropoietin concentrated solution.[18]

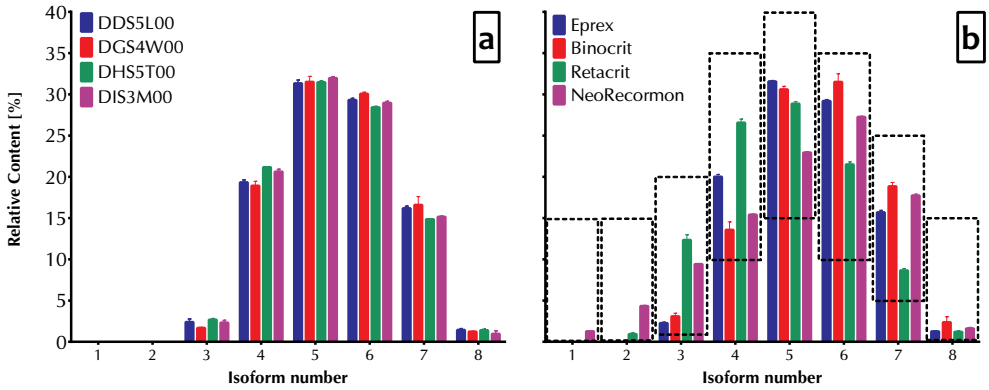

Figure 7. Relative isoform distribution of (**a**) different batches of Eprex (n=3) and (**b**) different epoetin products, namely Eprex (n=12), Binocrit (n=8), Retacrit (n=13), and NeoRecormon (n=10). The area inside black box represents the acceptance criteria based on Ph. Eur. monograph on Erythropoietin concentrated solution. Error bar indicates standard deviation.

Potency

Potency was determined by both *in vitro* and *in vivo* bioassay, as summarized in **Table 4**. All tested products are within the specifications (80%-125%), as indicated by Ph. Eur. monograph on Erythropoietin concentrated solution for *in vivo* bioassay.[18] The observed differences in the *in vitro* or *in vivo* potency ratio are likely due to different manufacturing processes, leading to different glycoproteins with varying degrees of sialylation, thereby affecting *in vivo* and *in vitro* bioactivity.[31]

Table 4. Weighted Mean Potencies [IU/mL] of 4 Epoetin Products With Upper and Lower 95% Confidence Limits

Brand Name (INN)	Lot Number	Declared Potency [IU/ml]	Content UV280nm [IU/ml]				*In vitro*/ *in vivo*
			In vitro potency [IU/mL]		*In vitro* potency [IU/mL]		
Eprex (epoetin alfa)	DDS5L00	10,000	12,500	[12,100-13,000]	N.D.		N.D.
	DGS4W00		N.D.		N.D.		N.D.
	DHS5T00		N.D.		N.D.		N.D.
	DIS3M00		13,100	[12,700-13,500]	N.D.		N.D.
Binocrit (epoetin alfa)	341211	10,000	9803	[14,900-16,600]	9,395	[8,190-10,777]	1.84
	450112		9400	[14,300-16,400]	8,015	[6,828-9,408]	1.90
	730412		9553	[14,300-16,400]	8,544	[7,573-9,640]	1.63
Retacrit (epoetin alfa)	8K058L8	10,000	8,610	[7,880-9,410]	9920	[8,234-11,951]	0.87
	8M072C9		9,190	[8,300-10,200]	N.D.		N.D.
	9F081G9		9,440	[9,000-9,900]	N.D.		N.D.
	9M108N9		9,040	[8,680-9,400]	11,886	[9,834-14,365]	0.76
NeoRecormon (epoetin beta)	H0002H01	50,000	54,000	[52,500-55,500]	49,483	[42,123-58,128]	1.09
	H0003H01		57,500	[55,500-59,500]	50,965	[43,401-59,847]	1.13

N.D., not determined

DISCUSSION

Most previous studies have shown the physicochemical properties of a single batch of multiple epoetin brands.[9,10,14] A few other studies have focused on the biological properties of different batches of a single epoetin product.[13,16] In contrast, we conducted physicochemical characterization and potency studies of the original and biosimilars in the European market including multiple batches. We found that products tended to differ in content, isoform profile, and potency. We also observed differences in content between different batches of the same product.

The use of different nonionic surfactants, PS20 and PS80, to minimize surface adsorption and stabilize protein from aggregating may contribute to the discrepancies in content.[29] As an earlier study has shown, detectable "HMW species" in the presence of PS80 implies the formation of micellar epoetin in Eprex, a possible explanation of the upsurge in PRCA incidence in 1998.[25] Another possible explanation is that PS80 causes leaching, which in turn elicits antibody-mediated PRCA.[6] To date, the exact interaction between PS80 and epoetin or other buffer components is still not completely understood. Although the debate on the plausible PRCA factors is still ongoing, the increasing use of PS in biologicals should be monitored for any possible chemical degradation leading to formation of acids and peroxides.[32] By keeping nonionic surfactants at the lowest possible amount in formulation, the protein damage can be minimized. Nevertheless, in this study, no epoetin–associated HMW was detected, suggesting low-immunogenic risk in the tested products.

In addition to a possible HMW species–induced immune response, immunogenic properties might be affected by glycosylation,[33] which also alters potency, that is, for epoetin.[15] Owing to variation in purification and different host cell used to produce protein, this post-translational modification was shown to be heterogenic between types of epoetin but homogenous among batches of single product assessed in this study.

Here, differences in the ratio of *in vitro* and *in vivo* potency between tested epoetins were observed but, not surprisingly, were less striking when comparing different batches of the same product. Although third WHO IS for erythropoietin may also vary, this indicates that different manufacturing processes will alter the degree of sialylation in epoetin products. Increases in the degree of terminal sialylation are known to correlate with increased bioavailability *in vivo.*[31] However, these effects are reversed *in vitro* presumably as a result of the reduced binding affinity of highly sialylated epoetin products to target cell receptors as a consequence of increased steric hindrance.

We exhibited that the epoetin products differ in some aspects of the quality attributes. Some of these differences may be induced during storage between the time of manufacturing and analysis. Certainly, this reflects the clinical situation because products are commonly stored for a certain period of time before being administered to patients. These differences, however, seem not to be clinically meaningful as long as the products are used within their shelf life, as demonstrated by several clinical studies.[34–38] Moving forward, establishing a link between analytical and clinical data of the same products (and batches) might benefit the future development of biosimilars.

CONCLUSIONS

The quality of the 4 epoetin products, each with multiple batches, is high. At the same time, differences in content, isoform profiles, and potency were observed not only in products from different manufacturers but also in different batches of the same product. Such variations in quality attributes are unavoidable because epoetin manufacture requires the use of living cells, in line with the "similar but not identical" paradigm.[39] Hence, being different from the innovator does not necessarily imply inferior product quality. This is something clinicians, pharmacists, and patients will need to understand. For now, the regular physiochemical characterization and biological assay remain crucial to identifying whether deviation in these products should require additional data on the preclinical and clinical level.[40]

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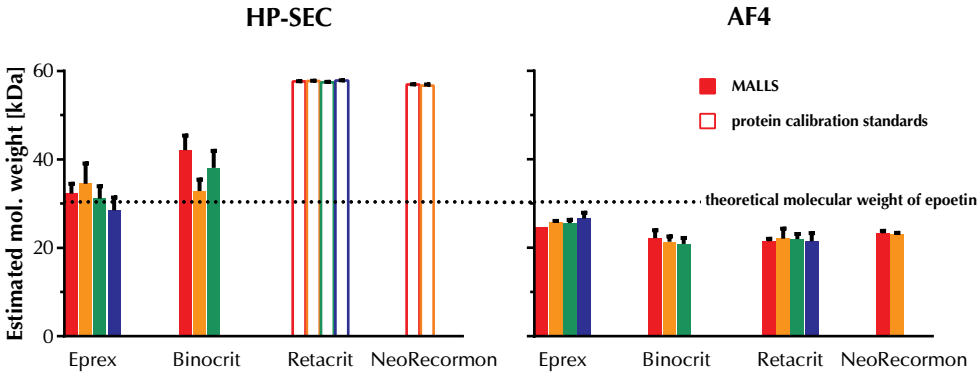
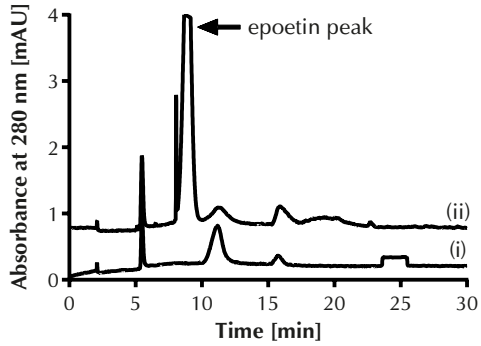
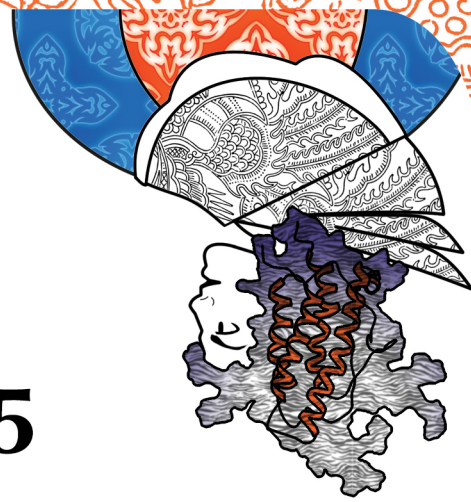
APPENDIXES



Supplementary Figure 1. The estimated molecular weight of tested epoetin products determined by either MALLS or calibration standards of proteins with known molecular weight. Different colors represent different batches. Error bar indicates standard deviation.



Supplementary Figure 2. Zoomed AF4 elugrams of (i) 0.3 mg/mL PS80 and (ii) Eprex batch DIS3M00.



Chapter 5

How Bio-questionable are the Different Recombinant Human Erythropoietin Copy Products in Thailand?

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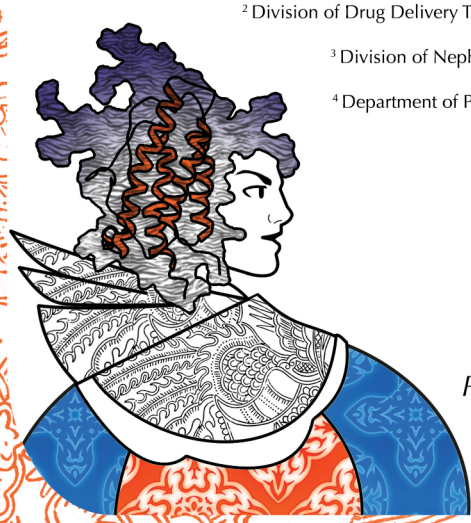

Pharm Res. 2014 May;31(5):1210-8

ABSTRACT

Purpose The high prevalence of pure red cell aplasia in Thailand has been associated with the sharp increase in number of recombinant human erythropoietin (rhEPO) copy products, based on a classical generic regulatory pathway, which have entered the market. This study aims to assess the quality of rhEPO copy products being used in Thailand.

Methods Twelve rhEPO copy products were purchased from pharmacies in Thailand, shipped under controlled cold chain conditions to the Netherlands and characterized using (1) high–performance size–exclusion chromatography, (2) asymmetrical flow field–flow fractionation, (3) sodium dodecyl sulfate–polyacrylamide gel electrophoresis in combination with (4) western blotting and additionally tested for (5) host cell protein impurities as well as (6) endotoxin contamination.

Results Some of the tested rhEPO copy products showed high aggregate levels and contained a substantial amount of protein fragments. Also, one of rhEPO copy products had a high endotoxin level, exceeding the FDA limit.

Conclusions Our observations show that some of the tested copy products on the Thai market differ significantly from the originator rhEPO product, Epogen®. This comparison study supports a link between the quality attributes of copy rhEPO products and their immunogenicity.

KEY WORDS *biosimilar, immunogenicity, protein characterization, pure red cell aplasia, recombinant human erythropoietin*

INTRODUCTION

Recombinant human erythropoietin (rhEPO) is used worldwide to treat anemia in patients with chronic kidney disease. Like all other therapeutic proteins, rhEPO may show immunogenicity. Although the occurrence of antidrug antibodies in patients is rare, if they are formed, they may cross-react with endogenous EPO and may lead to pure red cell aplasia (PRCA). Between 1998 and 2002, such an outbreak of anti-rhEPO antibody–induced PRCA took place.[1]

Although the exact mechanisms underlying antibody-induced PRCA remain unknown, the increased incidence of PRCA has apparently been contributed to by the replacement of human serum albumin (HSA) by polysorbate 80 and glycine as stabilizer, and by the use of uncoated stoppers in Eprex®.[2-4] More recently, PRCA has been attributed to tungsten-induced denaturation and aggregation of rhEPO.[5] Overall it appears that product quality is the main determinant of the immunogenicity of rhEPO.

In addition to the originator rhEPO products, rhEPO biosimilars strictly by criteria evaluation or as intended copy products are available.[6,7] Because the European Medicines Agency and the Food and Drug Administration (FDA) recognize the complexity of these products and consider generic drug approval guidelines unsuitable for market authorization, quality is ensured with tailored biosimilar guidelines that guarantee only products able to match the quality, efficacy, and safety of the originator product get market approval.[8,9]

However, in developing countries such as Thailand, where biosimilar guidelines are still under development, rhEPO copy products have entered the market under a generic drug paradigm. Dozens of rhEPO copy products are on the Thai market, registered as generic products.[10] Additionally, an increased incidence of PRCA in the Thai population has been shown by several reports.[10,11] This increase in PRCA in Thailand might be due to a different genetic background of the patient population,[12] or alternatively could be due to different product quality attributes.[6,7,13] The latter has not yet been studied.

We compared the quality of 12 different copy rhEPO alpha products containing HSA available on the Thai drug market to the originator rhEPO alpha product, Epogen®. We employed high–performance size–exclusion chromatography (HP–SEC) and asymmetrical flow field–flow fractionation (AF4) to assess monomer and aggregate content, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in combination with Western blotting to detect fragments and covalent protein aggregates. Finally, we determined the level of host cell protein impurities in these products as well as endotoxin contamination.

MATERIALS AND METHODS

RhEPO Products

Twelve different copy rhEPO alpha products manufactured worldwide were collected from one specific region in Thailand Thailand. As listed in **Table 1**, all available copy products were purchased from pharmacies in Bangkok, Thailand, and shipped via a certified courier to Utrecht University, the Netherlands. Products were temperature monitored during shipping to ensure cold

chain. Upon arrival, products were stored at 2–8°C and handled according to the manufacturers' specifications. Each product was inspected visually at the lab bench before analysis to check for the presence (if any) of visible particulates, since the products should be clear, colorless solutions.

All testing was performed before the expiry date. Per product, one batch was used to compare its quality to the originator rhEPO product, Epogen® (Amgen, Thousand Oaks, California, USA). As all the tested copy products do contain HSA, Epogen® was considered a more suitable control than Eprex®. Epogen® still contains HSA in its formulation; both contain a comparable rhEPO alpha drug substance developed by Amgen and the two brand names indicate only that they are marketed by two different companies.

Table 1. Origin and Declared Potency of Copy rhEPO Alpha Products Used in the Current Study. One Batch per Product was Used

10,000 international units/syringe	Korea 1
4,000 international units/syringe	China 1, China 2, China 3, India 1, Korea 2, Korea 3, Korea 4, Latin America 1, Latin America 2, Latin America 3 and Latin America 4

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

To visualize proteins (including fragments and covalent aggregates), SDS–PAGE was performed under non-reducing and reducing conditions. Per rhEPO product, an amount corresponding to 2 IU was mixed with 1x NuPAGE® lithium dodecyl sulfate sample buffer with or without NuPAGE® Reducing Agent containing 50 mM dithiothreitol to a final volume of 12 µL. All samples were subsequently heated at 70°C for 10 min and loaded (10 µL) on a NuPAGE® Novex® 4–12% Bis-Tris Gel. The PAGERuler prestained protein ladder (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used as molecular weight standard. Separation was performed at a constant voltage of 70 V for 30 min followed by 150 V for 60 min. After separation, proteins were visualized with a SilverQuest™ Silver Staining Kit. Unless stated otherwise, all products used were obtained from Life Technologies (Carlsbad, California, USA).

Western Blotting

After SDS–PAGE (described above), samples were transferred to an iBlot® Transfer Stack (nitrocellulose membrane, 0.2 µm) using the iBlot® 7-Minute Blotting System (Life Technologies, Carlsbad, California, USA) following the manufacturer's recommendations. The membrane was incubated in blocking buffer (5% non-fat dry milk [ELK, Campina Melkunie, the Netherlands] in 1xTBS-$T_{0.05}$ [50 mM TRIS–HCl, 150 mM sodium chloride and 0.05% (w/v) Tween 20, pH 7.4]) for 60 min at 25°C with constant orbital shaking. Following washing with 1xTBS-$T_{0.05}$, rhEPO or HSA was detected with a primary antibody directed against human EPO (custom-made rabbit polyclonal anti-EPO, 10 µg/mL, Biogenes, Germany) or an antibody directed against HSA (rabbit monoclonal anti-HSA, 1:5000, Bioke) in 5% bovine serum albumin by overnight incubation at 4ºC. The blot was washed four times for 5 min with 1xTBS-$T_{0.05}$ and then incubated with anti-rabbit (goat polyclonal, horse-radish peroxidase labeled, 1:100,000, Jackson ImmunoResearch,

West Grove, Pennsylvania) for 60 min at RT. Upon washing for four times for 5 min with 1xTBS-$T_{0.05}$, the blot was exposed to SuperSignal West Femto Maximum Sensitivity Substrate (1:2 in phosphate buffered saline, ThermoFisher Scientific, Waltham, Massachusetts, USA) for 10 s at room temperature. Bands were visualized with a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software (Bio-Rad, Hercules, California, USA).

High–Performance Size–Exclusion Chromatography (HP–SEC)

Prior to measurement, the rhEPO products were diluted to a concentration of 2,000 IU/mL using Milli-Q water. Subsequently, 100 IU per sample was loaded onto a Tricorn™ high performance column Superdex 200 10/300 GL (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) installed on a Waters 2695 Separations Module (Waters Corporation, Milford, Massachusetts, USA) at 30°C. Because Western blotting showed the presence of HSA in the rhEPO products, both Erythropoietin Biological Reference Preparation (EPO-BRP) batch 3 (the European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France) and human serum albumin (HSA, Sigma-Aldrich, St. Louis, Missouri, USA) were included as controls. Five different concentrations, ranging from 2.5 to 0.03 mg/mL of HSA and rhEPO were taken. The running buffer was composed of 1.5 mM potassium phosphate, 8.1 mM sodium phosphate and 0.4 M sodium chloride at a pH 7.4 and filtered through a 0.2 µm filter (Sigma-Aldrich, St. Louis, Missouri, USA) prior to use. Detection took place with a Waters 2487 Dual λ Absorbance Detector (Waters Corporation, Milford, Massachusetts, USA) at 280 nm. A gel filtration calibration kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was used to acquire a calibration curve. Data was collected and analyzed using the Empower 2 software version 6.20.00.00.

Asymmetrical Flow Field–Flow Fractionation (AF4)

In contrast to HP–SEC, AF4 is a matrix-free technique that uses an asymmetric cross flow to analyze proteins based on molecular weight. Proteins were separated on an Agilent 1200 apparatus (Agilent Technologies, Palo Alto, California, USA) with a UV detector (280 nm) and combined with a multiangle laser light scattering (MALLS) detector DAWN® HELEOS™ (Wyatt Technology Europe GmbH, Dernbach, Germany). Prior to measurement, all rhEPO products were diluted to 2,000 IU/mL in MilliQ-water. Fifty microliters of all rhEPO products were injected through a channel equipped with a 350 µm spacer of medium width and a regenerated cellulose membrane with a cutoff of 5 kDa. The detector flow and the focus flow were set to 1 mL/min and 1.5 mL/min, respectively. The elution settings used are summarized in **Table 2**. The running buffer was composed of 8.1 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4 and 0.4 M NaCl at pH 7.4 (Sigma-Aldrich, St. Louis, Missouri, USA) and filtered through a 0.2 µm-pore polycarbonate filter (GE Osmonics Inc., Minnetonka, Minnesota, USA) before use.

Table 2. AF4 Elution Program Settings

	Start [min]	Duration [min]	Cross-flow [mL/min]
Elution	0	2	1.8
Focus	2	1	1.5
Focus + injection	3	2	1.5
Elution	5	10	1.8
Elution	15	10	0

The root mean square (rms) diameter was calculated from the MALLS signal, using the Berry Fit option of the Astra software version 5.3.2.22 (Wyatt Technology Europe GmbH, Dernbach, Germany).

Host Cell Protein (HCP) Analysis

To quantify traces of Chinese hamster ovary (CHO) proteins in the rhEPO products, a CHO HCP ELISA Kit (Cygnus Technology, Southport, North Carolina, USA) was used. In short, 50 µL of the rhEPO products were placed in duplicate onto a 96-well plate pre-coated with affinity purified capture goat polyclonal anti-CHO antibodies. Samples were incubated with HRP-labeled goat polyclonal anti-CHO antibodies for 2 h at room temperature with constant orbital shaking (180 rpm). The supernatant was discarded and the plate was washed 4 times with 1x washing buffer (TRIS–buffered saline). Upon washing, plates were developed with 3,3′,5,5′–tetramethylbenzidine substrate for 30 min without shaking. Development was stopped by adding 0.5 N sulfuric acid. Optical density was measured at 450/650 nm using SPECTROstar® Nano (BMG LABTECH GmbH, Ortenberg, Germany). Values were corrected for background and a standard curve was constructed using the supplied CHO HCP standard. Data was fitted to a 4–parameter logistic fit using GraphPad. Subsequently, HCP content in each rhEPO formulation was determined.

Limulus Amebocyte Lysate Endotoxin Assay

Endotoxin levels of the rhEPO products, including control Epogen®, were assessed with a colorimetric Limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland). All measurements were performed by Lonza (Verviers, Belgium) and samples were temperature-controlled transported to Lonza using a certified courier. The assay procedure was performed as follows. First rhEPO samples were diluted ten times in LAL reagent water, followed by heat treatment at 75°C for 30 min to inactivate HSA. Per product, a volume of 100 µL of a 1:100 dilution was dispensed onto a microplate well in duplicate. To verify that the products themselves would not interfere with endotoxin measurements, an additional plate was prepared using the same dilutions (duplicate) of each rhEPO copy product, and 0.5 endotoxin unit per milliliter (EU/mL) of *Escherichia coli* (*E. coli*) endotoxin stock solution was used to spike into the samples. The plates (spiked and unspiked) were tapped gently on the side repeatedly to facilitate mixing followed by incubation at 37°C for 10 min. Then, 100 µL of lysate solution was added to each well and incubated for an additional 10 min. Absorbance was immediately read at 405–410 nm using a Lonza Kinetic QCL Reader BE25-315 with WinKQC software. Distilled water was used to adjust

the photometer to zero absorbance. A standard curve with the best-fit straight line was generated using an *E. coli* endotoxin standard prepared at five different concentrations (50 EU/mL–0.005 EU/mL). The endotoxin concentration in each of the rhEPO samples was calculated based on the generated equation.

RESULTS AND DISCUSSION

Prior to characterization, all rhEPO products were examined visually. All products were clear and colorless solutions and contained no visible particulates.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The molecular mass distribution of proteins was first investigated by SDS–PAGE. As shown in **Figure 1a**, under non-reducing conditions, all products including controls displayed a band corresponding to monomeric rhEPO (molecular weight, 30.4 kDa) at an apparent size of 34–38 kDa. This slight difference between expected molecular weight and the weight indicated by the band on SDS–PAGE is consistent with previous reports.[14-19] Principally, it is due to insufficient binding of SDS and lack of proper micelle formation.[20] The thickness of the rhEPO monomer band was quite variable between products, suggesting difference in mass distribution and/or in the structure of glycoforms.[21] In addition to the band corresponding to rhEPO monomer, all products showed another strong band at 50–60 kDa, which suggests the presence of HSA in their formulations. Above 80 kDa, a high molecular weight (HMW), additional bands/smears were present for all rhEPO copy products and for the originator rhEPO product, Epogen®. The pattern of this band/smear was similar to the HSA control indicating that the rhEPO products might contain HMW species due to HSA. However, it is also possible that rhEPO was present in these HMW species. Bands below 30 kDa, as observed for all rhEPO copy products and Epogen®, showed the presence of fragments and small oligomers within these products.

In comparison to Epogen®, most rhEPO copy products had comparable bands/smears. However, rhEPO copy products China 3 and Latin America 2 showed the most pronounced HMW bands above 80 kDa. Extra bands above 65 kDa were observed for Korea 2 and Latin America 3. Compared to Epogen® and the other rhEPO copy products, India 1, Korea 2 and Latin America 2 showed additional bands below 30 kDa.

Under reducing conditions, at ~40 kDa, all products also show a visible band for rhEPO monomer (**Fig. 1b**). At HMW ranges, copy and control rhEPO products in general showed lighter tone smears compared to the smears found during non-reducing conditions, suggesting the breaking of HMW species. However, rhEPO copy products China 3 and Latin America 4 showed much darker tone smears, suggesting a higher amount of HMW fragments upon reduction. Inversely, all rhEPO copy products from Thailand and the control rhEPO showed darker tone smears below 65 kDa, suggesting the formation of fragments derived from either reduction of covalently linked aggregates or amino acid sequences that were cleaved off during manufacturing, handling and/or storage.

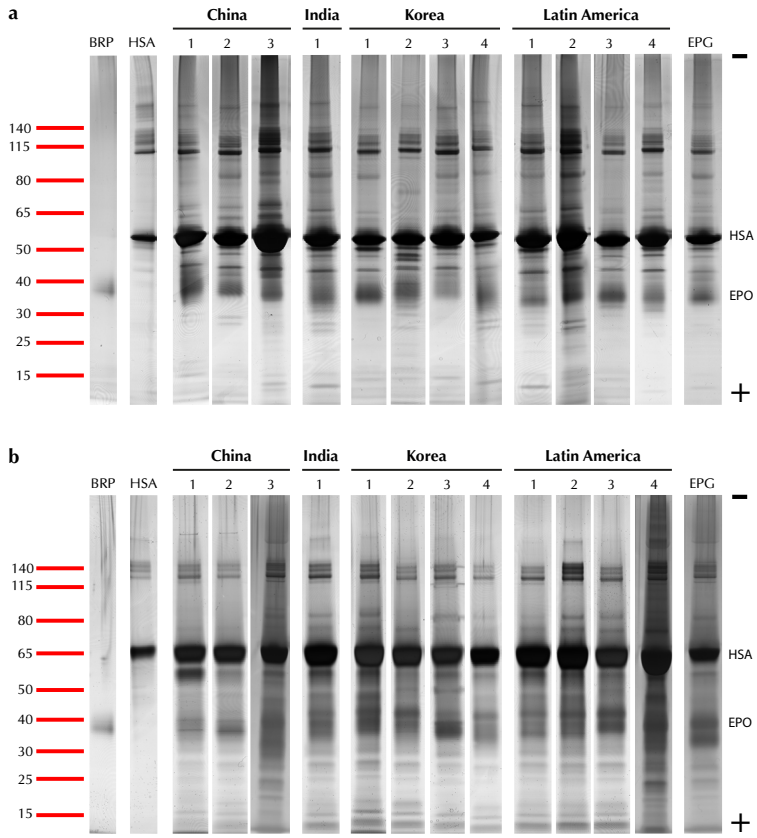

Figure 1. SDS–PAGE gel of different rhEPO copy products (2 IU/lane) stained with SilverQuest® Silver Staining under (**a**) non-reducing conditions and (**b**) reducing conditions. *Arrows* indicate the expected molecular weights of rhEPO or HSA. EPG stands for Epogen®.

Western Blotting

As indicated by the SDS–PAGE results, like the originator product, Epogen®, in their formulations, all rhEPO products appeared to contain HSA. The presence of HSA as well as EPO monomer, potential aggregates and fragments in the products were further assessed by Western blotting. Under non-reducing conditions, monomeric rhEPO was detected around 40 kDa (**Fig. 2a**), as previously observed for SDS-PAGE (**Fig 1a**). Additional bands just above 80 kDa were visible for all rhEPO copy products, in particular for Latin America 2. These bands suggest the formation of dimeric EPO-HSA, as confirmed by western blotting detected with anti-HSA antibody (**Fig. 3a**). in addition, rhEPO specific smears at HMW ranges were also visible for rhEPO copy product Latin America 2, indicative of rhEPO-containing aggregates.

In the presence of a reducing agent (**Fig. 2b**), most formulations showed two bands at ~40 kDa and 65 kDa, indicating the presence of monomeric and dimeric rhEPO, respectively. Only China 3 displayed no band at ~65 kDa. Smears at higher molecular weight range and the 80 kDa band were no longer present, suggesting the reduction of covalently linked aggregates and dimeric rhEPO-HSA observed during non-reducing conditions, respectively.

When using a specific anti-HSA antibody, bands at 50 kDa and 65 kDa were present for both non-reducing and reducing conditions (**Figs. 3a** and **3b**). This confirmed that, like Epogen®, all tested rhEPO products contained HSA. All products, including Epogen®, contained one or more additional bands with a molecular weight higher than 80 kDa, indicating the presence of aggregates containing HSA. Under non-reducing condition, the rhEPO copy product China 3 seemed to have the darkest tone bands in HMW region, indicating a higher concentration of HMW species as shown in **Figure 3a**. Besides HMW species, the rhEPO copy products China 3 as well as Latin America 2, 4 and Epogen® showed additional bands below the monomeric HSA band under reducing conditions (**Fig. 3B**), suggesting fragments as a result from either reduction of covalently linked aggregates or amino acids that were cleaved off during manufacturing, handling and storage. No band was observed in the EPO-BRP lane, indicating the specificity of the antibody (data not shown).

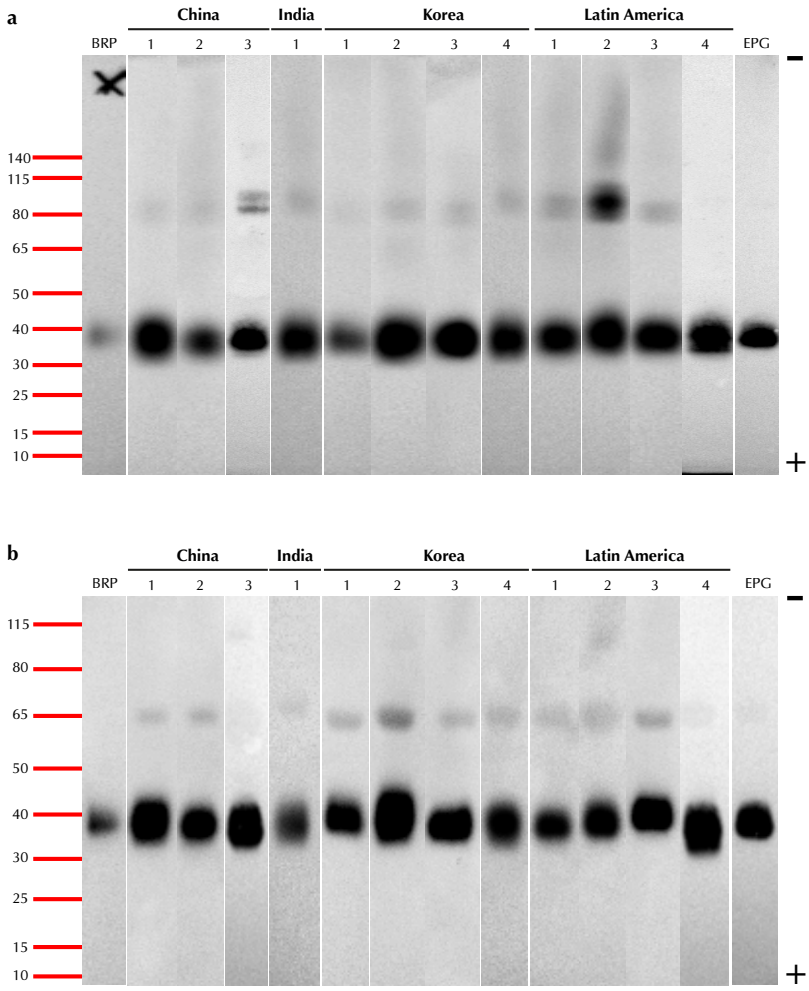

Figure 2. Performance of various rhEPO copy products on SDS–PAGE (2 IU/lane) detected with Western blot using anti-EPO antibody under (**a**) non-reducing and (**b**) reducing condition. EPG stands for Epogen®.

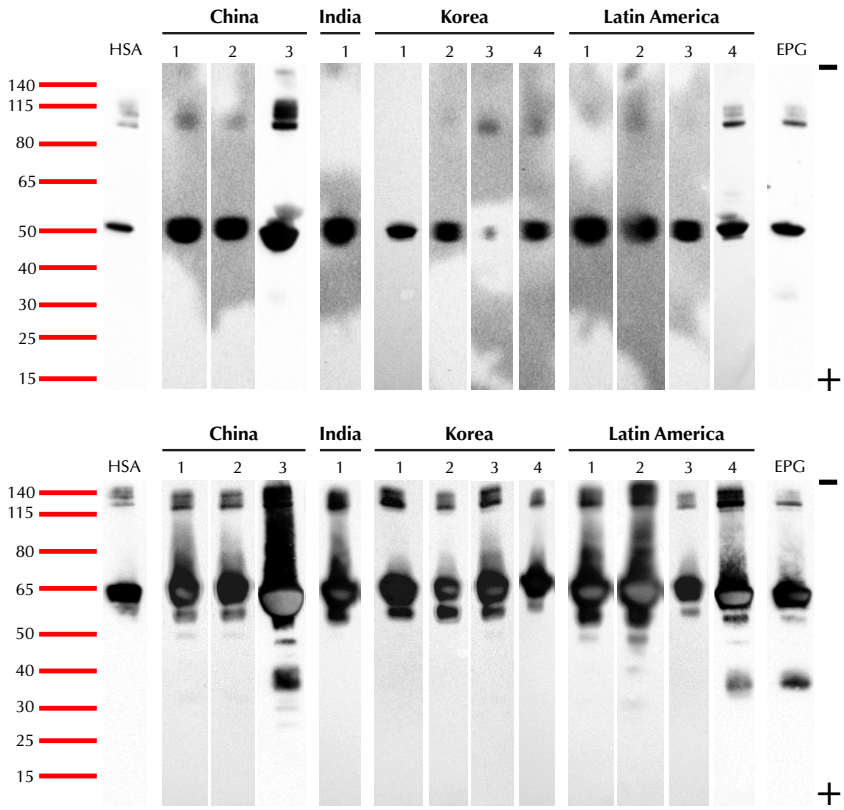

Figure 3. Performance of various rhEPO copy products on SDS–PAGE (2 IU/lane) detected with Western blot using anti-HSA antibody under (**a**) non-reducing and (**b**) reducing condition. EPG stands for Epogen®.

High–Performance Size–Exclusion Chromatography (HP–SEC)

All rhEPO formulations were assessed for protein composition using HP–SEC. As **Figure 4** shows, both rhEPO and HSA monomer co-eluted at a similar retention time. Because the rhEPO and HSA peaks were indiscriminate, we focused only on fragments and HMW species identified per product. The first peak, eluting at 16 min, indicates HMW species is absent in the EPO-BRP and HSA sample. HSA trimer, dimer and monomer were detected at 23, 25 and 29 min (**Fig. 4A**). In the HSA–free formulation *i.e.*, EPO-BRP, monomeric rhEPO was detected at 30 min. Meanwhile, a peak eluting at 29 min corresponded to the co-elution of between HSA and rhEPO monomers in all rhEPO copy products and Epogen®. Additional peaks eluting at 16 min and 24 min were possible rhEPO/HSA HMW and dimer, respectively. The other two peaks eluted at 39 min and 49 min were considered as low molecular weight species. They are likely formed due to formulation excipients and/or short amino acid sequences that might have cleaved off the rhEPO molecule. The chromatograms of all rhEPO copy products resemble Epogen®, except the rhEPO copy product Latin America 3, which differed from other products since a peak was visible at 45 min instead of 49 min.

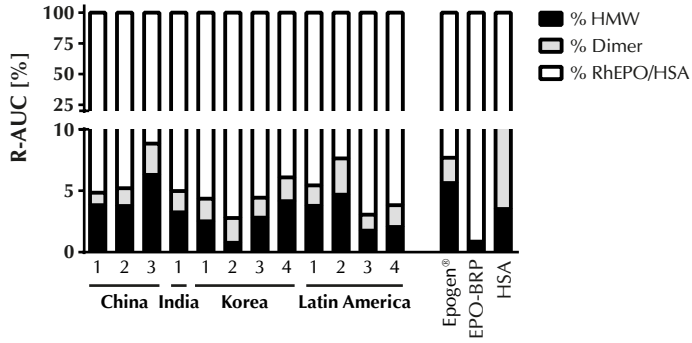

Figure 4. HP–SEC chromatograms of (**a**) HSA, EPO-BRP and Epogen®, and different rhEPO copy products from (**b**) China and India, (**c**) Korea and (**d**) Latin America, using UV detection at 280 nm. In all copy products and Epogen®, the dimer and HMW are likely to contain both rhEPO and/or HSA. The loaded amounts of EPO-BRP and HSA were 50 µg (~5706 IU) and 125 µg, respectively.

Figure 5 summarizes the areas under the curve (R-AUC) of these peaks relative to the total AUC for each product. Overall, most of the rhEPO copy products, compared to Epogen®, have a lower or similar percentage of HMW species. RhEPO copy product Korea 2 seems to contain the least amount of HMW species of all tested rhEPO products, while rhEPO copy product China 3 has the highest percentage (~4%). In addition, the R-AUC of low molecular weight species was also quantified in **Supplementary Figure 1**.

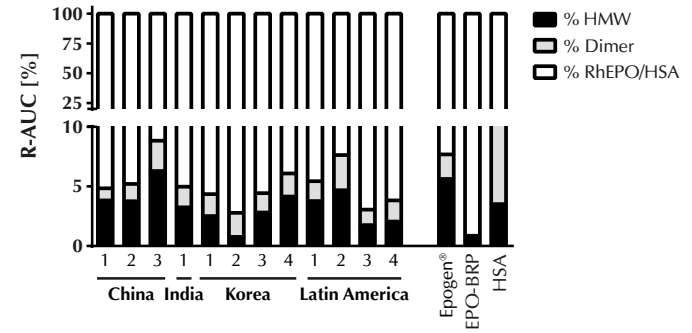

Figure 5. *Bars* represent the areas under the curve (R-AUC) of the HMW, dimer and rhEPO/HSA peaks relative to the total AUC for each product.

Asymmetrical Flow Field–Flow Fractionation (AF4)

AF4 was used as an orthogonal method to HP-SEC to assess the quality of the rhEPO products. Unfortunately, this analysis also showed the peaks corresponding to EPO monomer and HSA monomer as overlapping. Subsequently we focused on the presence of fragments and HMW species. **Figure 6** shows HMW species detected after the cross-flow was stopped at 15 min, and such protein species were present in all rhEPO copy products and Epogen®. Similar to HP–SEC, HMW species may contain both rhEPO and HSA. Interestingly, in control rhEPO, Epogen®, a distinct dimer peak was present in the co-elution peak of rhEPO and HSA monomer corresponding to possible HSA dimer. This peak was either (partly) present or absent for the other rhEPO copy

products. It might be due to differences in protein quantity and/or different dimers of rhEPO as well as HSA. It also seems that the higher the HSA content the more overlap of the monomer tail and dimer tail.

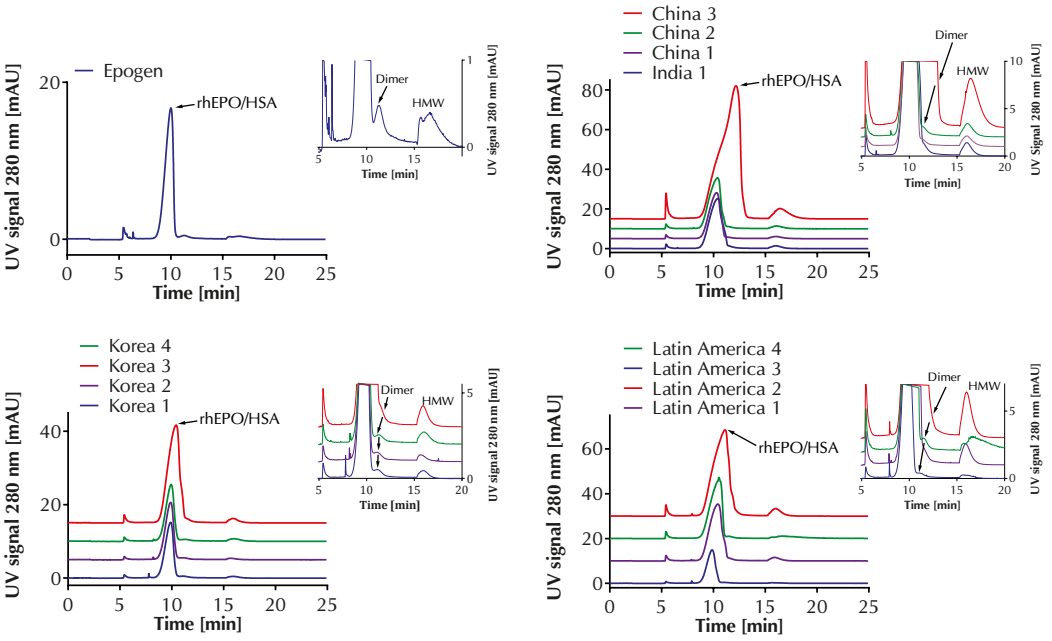

Figure 6. AF4 elugrams of (**a**) Epogen® and different rhEPO copy products from (**b**) China and India, (**c**) Korea and (**d**) Latin America using UV detection at 280 nm. In all rhEPO copy products and Epogen®, the dimer and HMW are likely to contain both rhEPO and HSA.

While rhEPO copy products China 3, Korea 4 and Latin America 4 stand out with a high percentage of HMW species (~6%), other copy and control rhEPO products contain 5% or less of HMW species (**Fig. 7**). The estimated molecular weight of these HMW species, MALLS analysis revealed, was between 0.8 and 4 x 10^3 kDa. The molecular weight of rhEPO/HSA as well as distinct dimer peaks were also estimated, as shown in **Supplementary Figure 2**. These data suggest that there are 3 rhEPO copy products with higher aggregate contents than other copy products and Epogen®, which might be a risk factor for immunogenicity.[22] When compared to HP–SEC data, this AF4 data seems to give a higher estimate of HMW species content (**Supplementary Fig. 3**). This, as Carpenter and colleagues have shown, may be because HP–SEC can underestimate the presence of HMW species too large to enter the matrix of the HP–SEC column.[23] Moreover, better recoveries of HMW species in AF4 than in HP–SEC have been previously observed.[24,25]

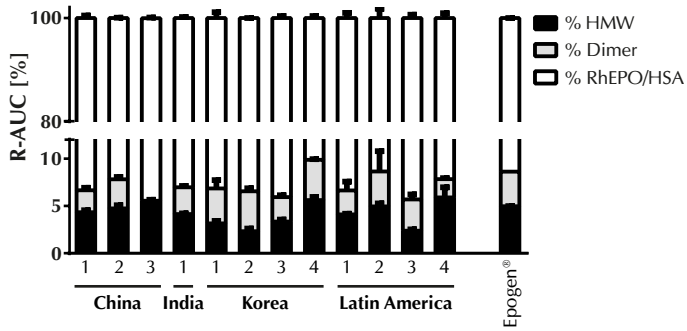

Figure 7. *Bars* represent the areas under the curve (R-AUC) of the HMW, distinct dimer and rhEPO/HSA peaks relative to the total AUC for each product.

Host Cell Protein (HCP)

Host cell impurities may compromise therapeutic efficacy of therapeutic proteins, including rhEPO, and may result in adverse effects such as immunogenicity.[26] As **Figure 8** shows, the amounts of CHO-derived proteins in the rhEPO products differ considerably. The product China 1 showed the highest level of HCP impurities while still remaining below the currently accepted limit of 100 ppm. Nevertheless, since a process or product specific HCP assays using anti-HCP antibodies specifically generated against the cell line or the product coming from a specific process was not available, the HCP values are most probably underestimated due to insufficient detection of all HCPs. Thus, elevated HCP values above the currently accepted limit of 100 ppm would be more meaningful than very low values as observed in this study.

Endotoxin Determination

Detecting endotoxin levels in (rhEPO copy) products is critical, as contaminants might be introduced during manufacturing, handling or storage. The presence of endotoxin in its drug formulation can result in pyrogenic responses and may affect immunogenicity of the finished products. For this reason, the endotoxin level was quantified in all rhEPO copy products and Epogen®. In most copy and control rhEPO products, endotoxin levels were below the detection limit of the assay. However, Latin America 4 had endotoxin levels exceeding the FDA limit (max. 0.5 EU/mL). This finding supports a previous publication by a group in Brazil in which 3 out of 12 rhEPO copy products from 5 different undisclosed suppliers showed substantially above FDA limits.[27]

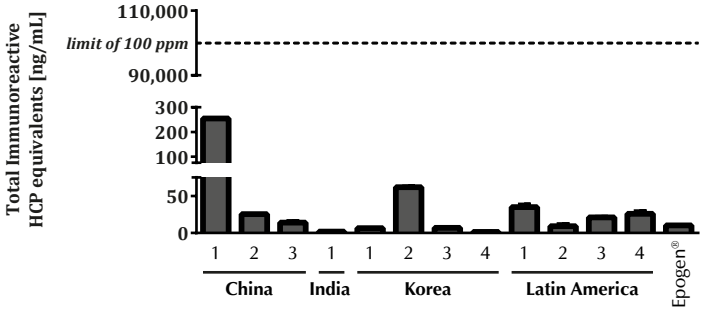

Figure 8. The quantification of host cell protein contained in different copy and control rhEPO products.

Gaps in Product Quality and Potential Safety

We have tested the quality of 12 different rhEPO products available on the Thai drug market. Although these products are used as more affordable and alternative treatment for renal anemia, their physicochemical properties show high aggregate rhEPO/HSA levels and contain a substantial amount of protein fragments for some of the rhEPO copy products. This could be a source of the higher risk of aggregate-induced immune response previously identified for these products.[28,29]

Besides possible aggregate–induced immunogenicity, bacterial endotoxin can induce the production of anti-rhEPO antibodies leading to, in some cases, PRCA.[30] In this study, we show that 1 out of 12 rhEPO copy products had high endotoxin levels, exceeding the FDA limit. Whether the differences mentioned above are correlated to the increasing PRCA incidence in Thailand remains speculative.[10,11]

The international consensus is that the generic pathway, as adopted by the Thai regulatory agencies, is not suitable for these copy products. Instead, the biosimilar pathway pioneered by the EU and adopted by the WHO would be beneficial, as both biological and clinical data of copy products should be present and should exhibit similar quality, safety and efficacy to the originator rhEPO product. At present, these copy products should not be considered as biosimilars.

CONCLUSION

To our knowledge, this is the first comparison study of different rhEPO copy products gathered from one specific region, Thailand. Batch-to-batch variability in addition to potency testing and glycosylation analysis will be included in future studies as part of the quality assessment of the rhEPO copy products. Our data is the first and part of a collaborative prospective study trying to understand the link between immunogenicity in Thai patients and product-related factors in rhEPO copy products on the Thai drug market. In combination with results on antidrug antibody incidences induced by these products (ongoing clinical study in Thailand), a link can hopefully be established. Until then, these rhEPO copy products remain bioquestionable.

ACKNOWLEDGEMENTS AND DISCLOSURES

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APPENDIXES

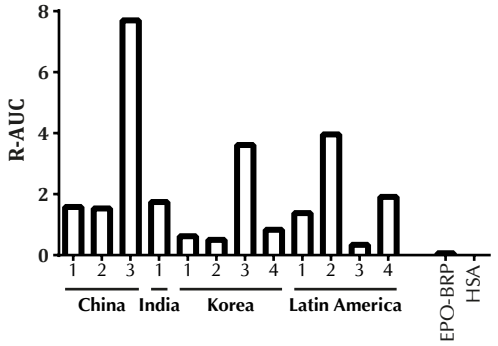

Figure S1. Relative AUC of the low-molecular-weight (LMW) fraction of all rhEPO copy products in comparison to control rhEPO, Epogen.

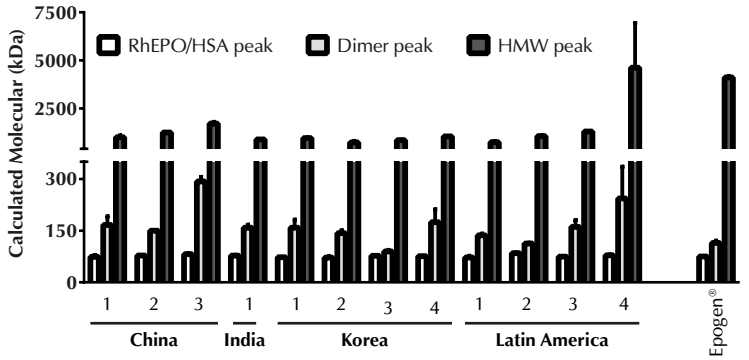

Figure S2. Calculated molecular weight based on UV and MALLS signal.

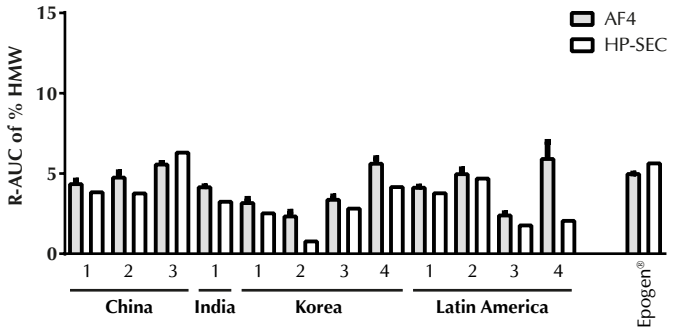

Figure S3. Bars represent the areas under the curve (R-AUC) of the HMW peaks relative to the total AUC for each product quantified by HP–SEC and AF4

Chapter 6

A Prospective, Immunogenicity Surveillance Registry of Recombinant Human Erythropoietin with Subcutaneous Exposure: Association with Product Quality in Thailand?

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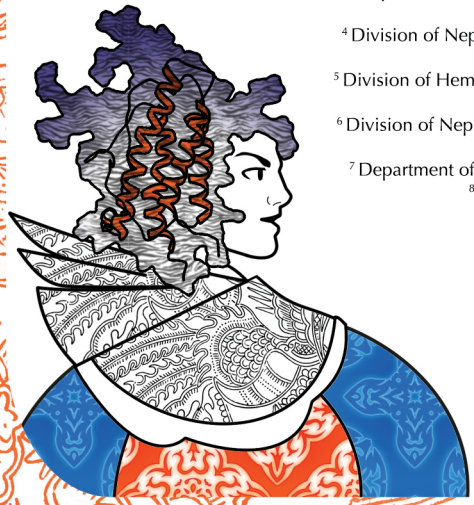

Manuscript in preparation

ABSTRACT

There have been increasing numbers of individual case reports of anti-recombinant human erythropoietin (r-HuEpo)–associated pure red cell aplasia (PRCA) by subcutaneous exposure in Thailand. To address the risk of anti-r-HuEpo–associated PRCA and to further elucidate the link between immunogenicity of r-HuEpo products and their quality, a prospective pharmacovigilance study and an assessment of product quality were performed. The incidence rate of anti-r-HuEpo–associated PRCA in this registry was 1.7 cases per 1,000 patients–year exposure. Of more than 20 r-HuEpo biocopy products currently licensed in Thailand, four brands were used in anti-r-HuEpo–associated PRCA cases. The quality studies of these four brands showed high aggregate levels and contained substantial amount of host cell proteins impurities. In addition, they also differed in isoform, amount of subvisible particles, and *in vitro* potency. Current study shows that the risk of r-HuEpo–associated PRCA among Thai patients is high and that there is an association between immunogenicity and quality of these biocopy r-HuEpo products.

INTRODUCTION

Pure red cell aplasia (PRCA) is a rare hematological disorder characterized by complete absence of erythroblasts in the bone marrow[1]. It may occur subsequent to the development of anti-erythropoietin antibodies. In addition to other factors, its incidence has been associated with the use of recombinant human erythropoietin (r-HuEpo) in which three PRCA cases were described between 1988 and 1998. The number of PRCA cases began to rise substantially and reached its peak in 2002 after slight formulation changes was introduced in Eprex®, an innovator r-HuEpo alfa, in 1998[2,3]. It began declining in 2003 after subcutaneous exposure was contraindicated and possibly after changes were made to the product in terms of rubber stopper coating.

At present, there are few reports of r-HuEpo–associated PRCA worldwide[4]; the exception is Thailand, where increasing numbers of individual cases of r-HuEpo–associated PRCA by subcutaneous (SC) exposure have been reported[5]. There are postulations that the immunogenicity of products may relate to human leukocyte antigen immunogenetic[6], storage and handlings[7] as well as formulations and quality of of r-HuEpo product[8].

The vast number of r-HuEpo products licensed for treatment of renal anemia in Thailand includes innovator products, Eprex®, Recormon®, Aranesp®, and Mircera®, and more than 20 biocopy products. The biocopy r-HuEpo products were approved using the classical generic drug paradigm, and did not require clinical study data or a full dossier[9]. With their usage, many clinicians in Thailand were starting to see an increase in PRCA cases, which raised the important issue of whether product qualities affect differences in immunogenicity between these biocopy r-HuEpo products and the innovator r-HuEpo. This raises the need for prospective pharmacovigilance and analytical study of products quality.

The Prospective Immunogenicity Surveillance Registry of r-HuEpo with SC exposure in Thailand aimed to estimate the incidence rate of r-HuEpo-associated PRCA among subjects who had SC exposure to any currently marketed r-HuEpo products. The registry was planned to first address the risk of anti-r-HuEpo–associated PRCA. Subsequently, it was also intended to investigate whether adverse immunogenicity could be related to product qualities.

RESULTS

Patient Characteristics

In the 4,018 cases enrolled in the registry, the average age was 57.4 ± 15.3 years (**Table 1**). The mean chronic kidney disease (CKD) diagnosis vintage before initiation of r-HuEpo was 18.6 ± 45.3 months. R-HuEpo products were prescribed for treatment of renal anemia for 99.8% of cases. The cause of CKD mirrored the etiology of CKD in Thailand, namely diabetic nephropathy (42.0%), hypertensive nephrosclerosis (34.9%), chronic glomerulonephritis (7.4%), obstructive uropathy (4%) and uric acid nephropathy (3.1%). More than 97% of cases were CKD stage IV and V. The average serum creatinine at enrollment was 9.02 ± 5.67 mg/dL and mean eGFR was 8.8 ± 7.9 mL/min/1.73m^2.

Table 1. Demographic data of the registry patients (n = 4,018)

Characteristics	
Male/Female (%/%)	49.9/50.1
Age: Mean(SD) (Year)	18.6 (45.3)
CKD Vintage: Mean (SD) (%)	57.4 (15.3)
History of kidney transplantation (%)	1.28
Clinical indication of ESA (%)	
Renal anemia	99.8
Myelodysplastic syndrome	0.2
Etiology of chronic kidney disease (%)	
Diabetic nephropathy	42
Hypertension	34.9
Chronic glomerulonephritis	7.4
Obstructive nephropathy	4
Uric acid nephropathy	3.1
Cystic kidney disease	1.3
Lupus nephritis	1.1
Analgesic nephropathy	0.5
Alport's syndrome	0.1
Allograft failure	0.1
Unknown	5.7
Stages of CKD (%)	
CKD stage 1	0.1
CKD stage 2	0.1
CKD stage 3	2.6
CKD stage 4	13.1
CKD stage 5	84.3
Serum creatinine: Mean (SD) (mg/dL)	9.02 (5.67)
Estimated GFR: Mean (SD) (mL/min/1,73m2)	8.8 (7.9)

There were 15 r-HuEpo products used in the registry including five innovator products (Recormon®, Eprex®, Erypo®, Mircera®, and Aranesp®) and ten biocopy products (Hypercrit®, Espogen®, Hemax®, Epiao®, Epokine®, Renogen®, Epoetin®, Bioyetin®, Eporon®, and Eritrogen®). Seven products were used in more than 90% of total cases in the registry, namely Hypercrit®, Espogen®, Hemax®, Recormon®, Eprex®, Epiao®, and Epokine®. The remainder of the products were used in between 1% and 5% of total cases. The wide variability in the use of r-HuEpo products in this registry (from less than 1% to more than 30% of total cases) and markedly small number of cases involving half of the products has meant comparison between products cannot be made with any certainty. In addition, although the inclusion criteria indicated SC exposure for subject enrollment, cases could be switched later on to intravenous administration by physicians. These represented fewer than 2% of cases overall.

ESA Treatment and Response

The pattern of r-HuEpo use over time shows that 80–85% of registered cases used a single r-HuEpo product for more than 36 months. Although the registry protocol indicated non-interventional study, the subjects tended to receive only a single r-HuEpo product throughout the study period. About 10–15% used two r-HuEpo products and 3–5% used three products (**Fig. 1**). The switching of r-HuEpo products was the result of changes in each hospital's drug formulary policy.

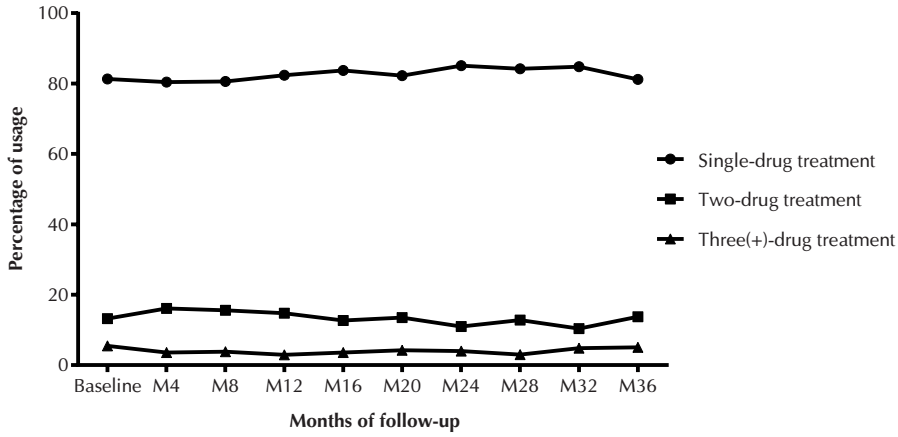

Figure 1. Percentage of patients with different ESA treatment over time.

The mean hemoglobin at baseline prior to initiation of r-HuEpo treatment was 9.2 ± 1.9 g/dL (**Fig. 2**). Following r-HuEpo treatment, the average hemoglobin increased to range between 10.2–10.9 g/dL at 36 months follow up. The mean r-HuEpo dosage excluding Aranesp® and Mircera® used by patients in this registry was 8,055 ± 4,801 units per week.

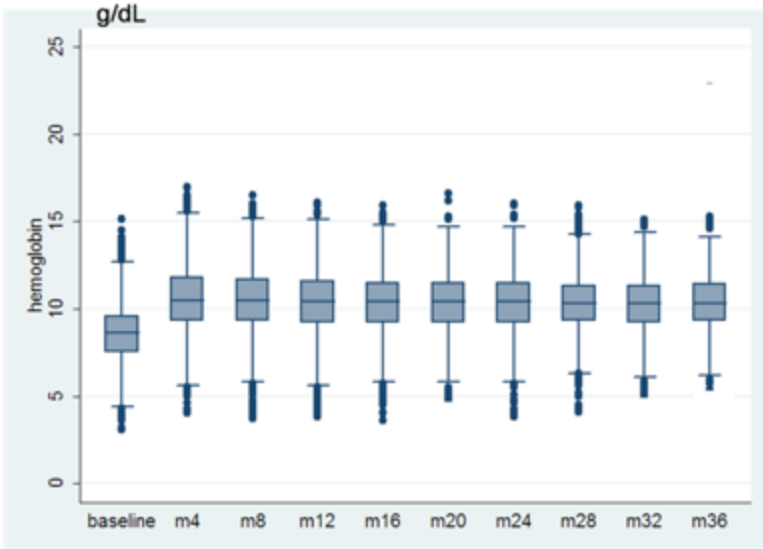

Figure 2. Hemoglobin response to r-HuEpo products.

Adverse Events

There were 78 adverse events in this registry (**Table 2**). Cardiovascular events were the most frequent. Cardiac disorders captured in the registry included acute coronary syndrome and congestive heart failure (**Table 3**). Other adverse events included infectious disorders such as bacterial sepsis, respiratory tract infections, gastrointestinal tract infections, vascular access infections, herpes zoster and peritonitis. Neoplasm disorders included bladder cancer, and prostate cancer.

Table 2. Adverse events by system organ class

System Organ Class Term	Frequency	% of disorder
Cardiac disorders	14	18
Infections and infestations	10	12.8
Renal and urinary disorders	9	11.5
Blood and lymphatic system disorders	9	11.5
Gastrointestinal disorders	9	11.5
Metabolism and nutrition disorders	8	10.3
Neoplasm benign, malignant	3	3.9
Reproductive system and breast disorder	2	2.6
Respiratory, thoracic disorder	2	2.6
Injury, poisoning and procedural complication	2	2.6
Vascular disorders	2	2.6
Nervous system disorders	2	2.6
General disorders	2	2.6
Hepatobiliary disorders	1	1.3
Immune system disorders	1	1.3
Endocrine disorders	1	1.3
Musculoskeletal and connective tissue disorder	1	1.3
Total	**78**	**100**

Table 3. Adverse events by preferred adverse term terms

Preferred adverse event term	Frequency	% of disorder
AKI on top CKD	8	10.3
Cardiac failure	8	10.3
Acute coronary syndrome	6	7.7
Worsening of anemia	6	7.7
Gastrointestinal hemorrhage	5	6.4
Pneumonia	4	5.1
Gastroenteritis	1	3.9
Hyperglycemia	3	3.9
Arteriovenous fistula complication	2	2.6
Cerebrovascular accident	2	2.6

Prostate cancer	2	2.6
Sepsis	2	2.6
Thrombocytopenia	2	2.6
Acute asthma	1	1.3
Bladder cancer	1	1.3
Bradycardia	1	1.3
Drug hypersensitivity	1	1.3
Fall	1	1.3
Glomerulonephritis	1	1.3
Hepatic encephalopathy	1	1.3
Herpes zoster	1	1.3
Hyperkalemia	1	1.3
Secondary hyperparathyroidism	1	1.3
Worsening hypertension	1	1.3
Hypoglycemia	1	1.3
Intestinal obstruction	1	1.3
Joint swelling	1	1.3
Edema	1	1.3
Pulmonary embolism	1	1.3
Peritonitis	1	1.3
Pyrexia	1	1.3
Thalassemia	1	1.3
Type 2 diabetes mellitus	1	1.3
Urinary tract infection	1	1.3
Uterine inflammation	1	1.3
Vaginal hemorrhage	1	1.3
Vitamin D deficiency	1	1.3
Total	**78**	**100**

Unexplained Loss of Efficacy

There were 9 cases of unexplained loss of efficacy (LOE) caused by anti-r-HuEpo–associated PRCA. The mean age was 52.7 ± 14.3 years. All of cases were late CKD and received r-HuEpo for treatment of renal anemia. The bone marrow biopsy of all cases revealed normocellularity with normal myeloid and megakaryocytic lineages, but absence of the erythroid precursor (<5% of erythroblasts in the bone marrow). The average r-HuEpo SC exposure duration was 13.6 ± 3.1 months. The range of r-HuEpo exposure time of anti-r-HuEpo cases was 3–21 months (**Table 4**). There were no cases developed immunogenicity after exposure of 21 months (**Fig. 3**). All of anti-r-HuEpo–associated PRCA cases received biocopy r-HuEpo products.

Table 4. Characteristics of anti-r-HuEpo–associated PRCA cases

Characteristics	
N (cases)	9
Male: Female (case/cases)	8/1
Age: Mean (SD) (years)	52.7 (14.3)
CKD vintage to enrollment: Mean (SD) (months)	11.8 (26.0)
Indication for r-HuEpo treatment	
Renal anemia (cases)	9
Etiology of CKD (cases)	
Diabetic nephropathy	3
Hypertensive nephropathy	3
Obstructive nephropathy	1
Uric acid nephropathy	1
Unknown	1
Serum Cr at enrollment: Mean (SD) (mg/dL)	13.37 (6.13)
eGFR at enrollment: Mean (SD) (mL/mim/1.73 m2)	4.44(1.42)
Exposure duration to r-HuEpo: Mean (SD)	13.6(3.1) (months)

R-HuEpo Biocopy Products Quality

Four r-HuEpo biocopy brands (brand A, B, C, and D) were used in anti-r-HuEpo–associated PRCA cases. One or more batches of these brands were evaluated by analytical methods as well as *in vitro* bioassay, and, if possible, compared to Eprex®, which is a human serum albumin (HSA) free formulation. All biocopy products used HSA for preservation.

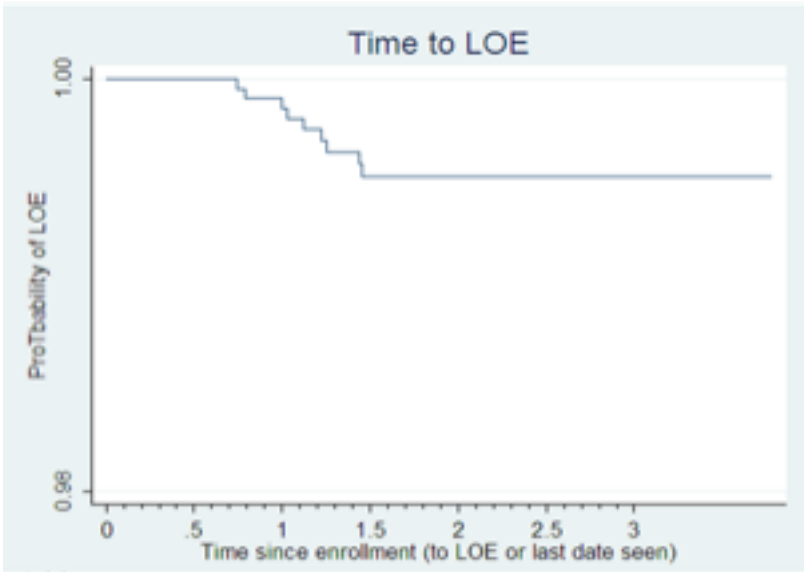

Figure 3. Overall Survival Function of LOE over time since enrollment.

The average hemoglobin of anti-r-HuEpo–associated PRCA cases at the unexplained LOE event was 5.3 g/dL (**Table 5**). All patients were blood transfusion dependent for relief of anemic symptoms. No cases had evidence of thymoma, cancer or autoimmune disease at the time of the unexplained LOE. The incidence rate of anti-r-HuEpo–associated PRCA in this registry was 0.0017 per person–year (95% confidential interval of 0.0007-0.0032) or 1.7 cases per 1,000 patient-year exposure (**Table 6**).

Table 5. Laboratory data of the PRCA patients (n=9)

Case	WBC (count/μL)	Hb (g/dL)	MCV	Platelet (cell/μL)
1	4880	6.6	70	187,000
2	5400	2.5	69	184,000
3	5600	6.1	76	125,000
4	6900	4.2	82	391,000
5	12200	5.4	82	216,000
6	5700	4.9	81	284,000
7	5600	3.9	73	124,000
8	7300	7.1	77	186,000
9	9300	7.4	85	409,000

Table 6. Incidence rate of unexplained LOE

Number of Cases	Time from initial diagnosis (Person-Years)	Incidence rate [95%Conf. Interval]
9	5268.29	0.0017083 [0.0007812 - 0.003243]

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis in Combination with Western Blotting

Under non-reducing conditions, monomeric r-HuEpo was detected at slightly below 40 kDa (**Fig. 4a**). This is in line with previous research, in which r-HuEpo with a molecular mass of 30.4 kDa is shown to migrate with an apparent size of 34–38 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis[10]. In brands A and B, two additional bands were visible at slightly higher than 80 kDa, indicating the presence of dimeric r-HuEpo–r-HuEpo (~75 kDa) and dimeric r-HuEpo–HSA (~95 kDa) where HSA migrates with an apparent size of 55 kDa[8]. Traces of r-HuEpo at higher molecular weight (HMW) ranges (140 kDa and higher) were also visible, mostly for brands A and B and to a lesser extent brand D. These results show that these brands contained non-covalently linked aggregates of either r-HuEpo or r-HuEpo/HSA as traces of HSA were also detected at similar HMW ranges with anti-HSA antibody (image not shown). Dimer and HMW species–containing r-HuEpo were also presence in brand C but at much lower intensity. Only single band was detected in Eprex® indicating that HMW species was absent. In the presence of a reducing agent (**Fig. 4b**), all products contained only single band corresponding to monomeric r-HuEpo at ~40 kDa which is slightly higher than under non-reducing condition.

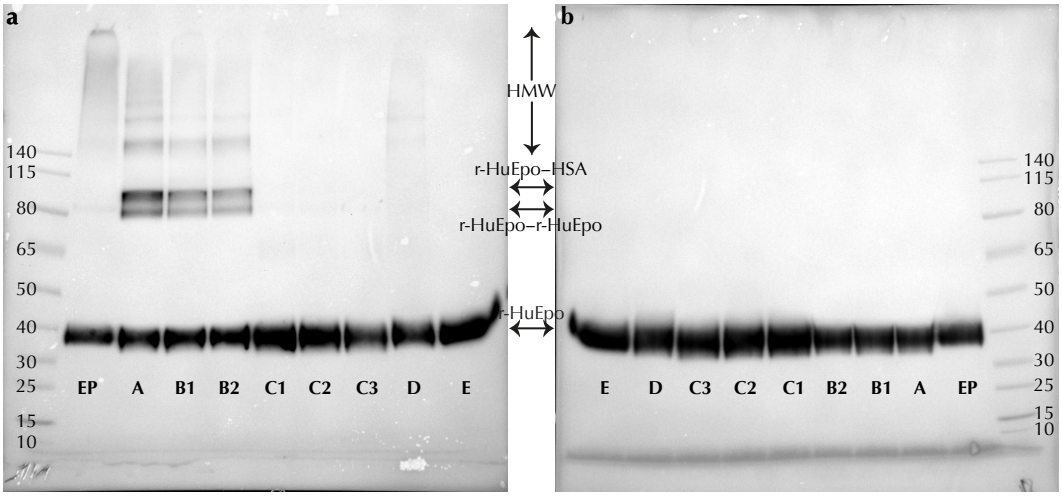

Figure 4. Performance of various r-HuEpo biocopy products on SDS–PAGE detected with Western blot using anti-r-HuEpo antibody under (**a**) non-reducing and (**b**) reducing condition. Different brands were indicated with letters A, B, C and D. Numbers represented different batch of one biocopy product. E indicates Eprex® and EP indicates Epogen® (stressed at 55°C for 14 days).

Iso–Electric Focusing in Combination with Western Blotting

Like other glycoproteins, r-HuEpo products contained mixtures of a multitude of isoforms, differing mainly in their glycosylation. **Figure 5** exhibits isoform patterns of four biocopy brands and Eprex. In some brands, differences in relative mobility (R_f), intensity, and number of isoforms were apparent compared to Eprex. While brand C contained seven isoforms similar to Eprex, brands A and B had one less isoform. Brand D had the lowest number of isoforms among all brands analyzed. Differences in R_f and number of isoforms were also observed between batches of brand B but were less striking in brand C.

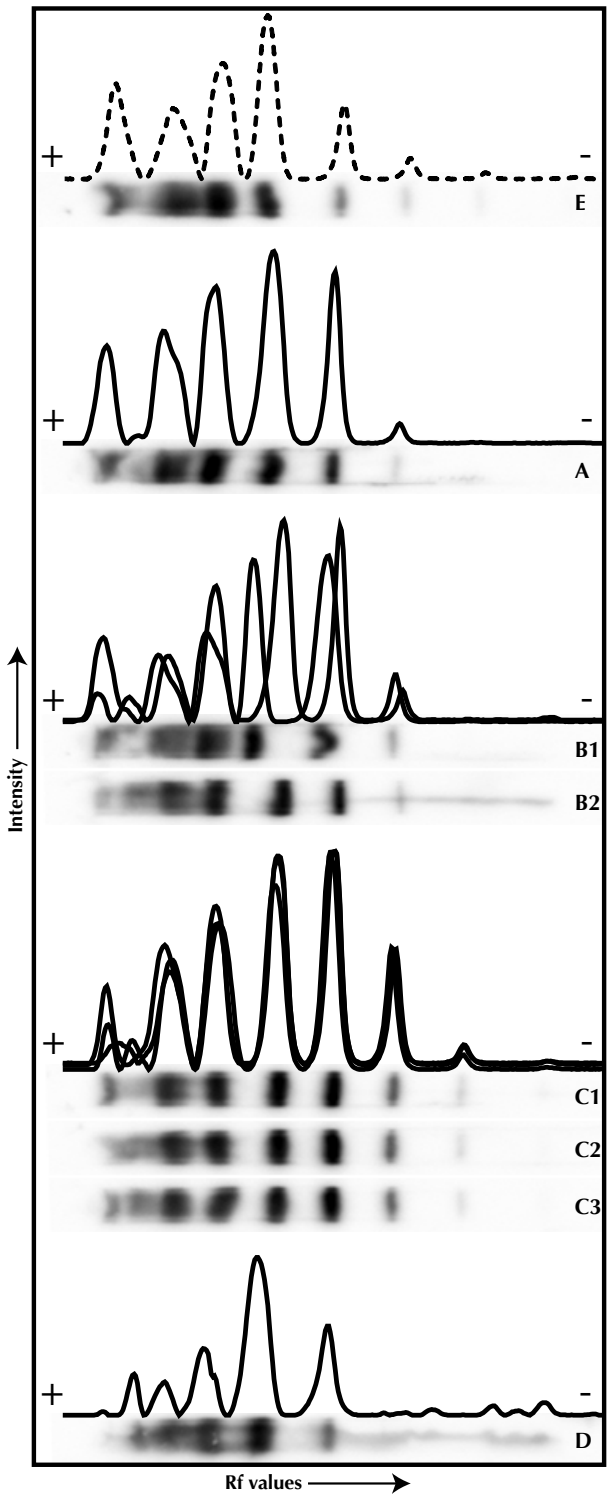

Figure 5. Performance of various r-HuEpo biocopy products on IEF detected with Western blot using anti-r-HuEpo antibody. Different brands were indicated with letter A, B, C and D. Numbers represented different batch of one biocopy product. E indicates Eprex. R_f indicates the relative mobility of the bands.

High–Performance Size–Exclusion Chromatography

Soluble aggregation in r-HuEpo biocopy products was assessed and quantified using high–performance size–exclusion chromatography. As **Figure 6** shows, both r-HuEpo and HSA monomer co-eluted at a similar retention time. Because the r-HuEpo and HSA peaks were overlapping, we focused only on fragments and HMW species identified per brand. In the formulation buffers of all brands except brand C, HSA trimer was detected at 14.7 min, dimer at 15.8 min, and monomer at 17.9 min. The r-HuEpo monomer in r-HuEpo chemical reference substances (CRS) was detected slightly later at 18.4 min (chromatogram not shown).

A peak eluting at 17.9 min corresponded to the co-elution between HSA and r-HuEpo monomers in all brands. Additional peaks eluting at 11.4 min and 13.5 min were HMW species. The other two apparent peaks eluted at ~24 min and ~28 min were considered to be low molecular weight species. They were likely formed due to formulation excipients and/or short amino acid sequences that might have cleaved off the r-HuEpo molecule. The chromatograms of all brands resemble each other, except brand D, in which a peak eluted at 28 min was absent.

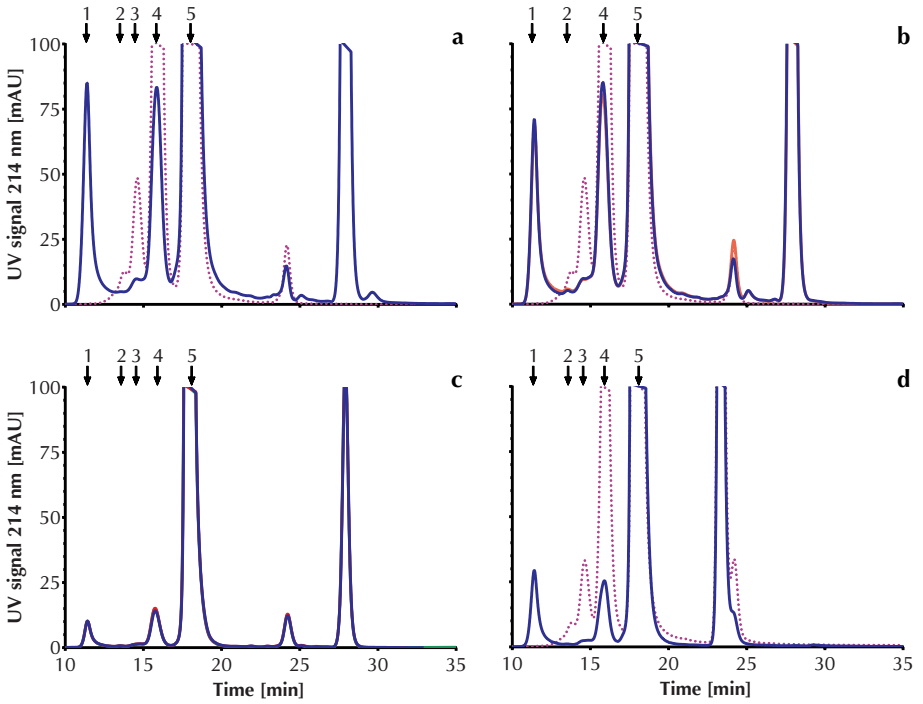

Figure 6. Zoomed HP–SEC chromatograms of one or more batches of different biocopy brands using UV detection at 214 nm. (1) HSA/r-HuEpo monomer, (2) HSA dimer, (3) HSA trimer, (4) HMW species 1, (5) HMW species 2. Different batches of the same product are shown in different colors. Dotted line indicated formulation buffer.

Subsequently, the total peak area of each population *e.g.* monomer, dimer and HMW species was calculated for each product and compared to the total peak area of all peaks in the chromatograms. As shown in **Figure 7**, the relative areas under the curve (R-AUC) of all populations were calculated for each product and expressed in percentage (%). Brand A contained the highest %HSA dimer, %HSA trimer, and %HMW species. Brand B had comparable %HSA dimer but an absence of HSA trimer and lower %HMW species compared to brand A. Meanwhile, brand C and D contained the least %HMW species.

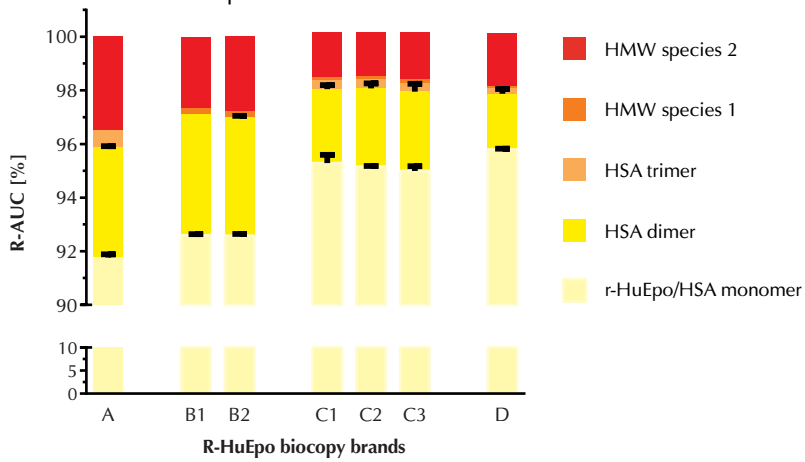

Figure 7. Bars represent the areas under the curve (R–AUC) of the HMW, distinct dimer and r-HuEpo/HSA peaks relative to the total AUC for each product.

Chinese Hamster Ovary Host Cell Proteins Impurities

Host cell protein (HCPs) impurities, if co-purified with the active protein, may trigger an immune response directed either against Chinese hamster ovary (CHO) HCP or against the active protein. Such a response can potentially lead to unwanted clinical consequences. **Figure 8** shows the amounts of CHO–derived HCP in all r-HuEpo biocopy brands. Brand D contained substantially higher amount of HCP impurities exceeding the typical purity targets *i.e.* 100 ng/mL[11]. Differences in the amount of HCP impurities were also observed between batches of the same brand. A twofold difference and more in the amount of HCP impurities were also apparent between batches of brand B and C.

With the exception of brand D, where the concentration of HCPs was underestimated at the 2- and 10-fold dilutions, the High Dose Hook Effect was investigated and showed not to affect the determination of HCPs content in tested brands (data not shown). In addition, recoveries in all brands were all within acceptable limits (80–120%) ranging from 94% to 113%. It is worth noting that since a process or product specific HCP assays using anti-HCP antibodies which were specifically generated against the cell line or the product coming from a specific process was not available, the HCP values may be underestimated due to insufficient detection of all HCPs.

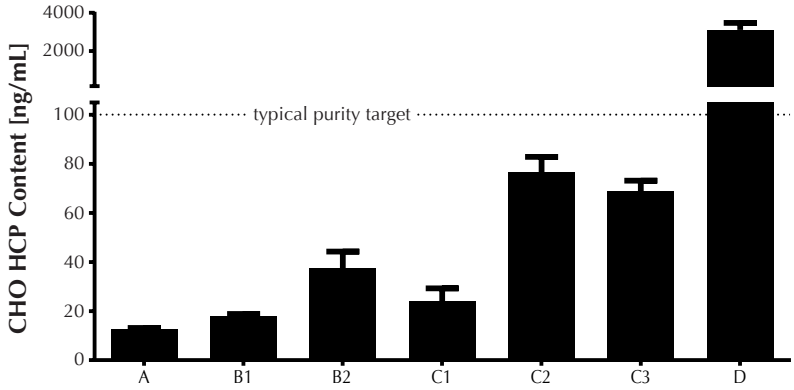

Figure 8. The quantification of CHO HCPs contained in different r-HuEpo biocopy products (n=8).

Endotoxin

The presence of endotoxin has been reported to interfere with therapeutic r-HuEpo in maintaining target hematocrit levels in the CKD patients receiving hemodialysis[12]. Hence, it is critical to quantify the amount of endotoxin in all r-HuEpo biocopy products. Our results indicate that endotoxin level in all brands was below the detection limit of the assay.

In Vitro Bioassay

Proliferation of erythroid progenitor cells induced by all r-HuEpo biocopy products was measured and compared to Eprex. We consider differences of more than 20% compared to Eprex as significantly different. As **Table 7** shows, brand B batch 2 showed significantly higher potency ~30% than of Eprex when tested at 1U EPO/boite. At 0.5U EPO/boite, even more biocopy products showed significantly higher potency than of Eprex. Two batches of brand C, one batch of brand B, and brand D were significantly more potent than Eprex ranging from ~25% to 40%.

Table 7. The *in vitro* bioassay of r-HuEpo performed in cultures of erythroid progenitor cells

R-HuEpo brands	batch	1U EPO/boite	0,5U EPO/boite
Eprex		412	305
A	1	442	292
B	1	465	412‡
	2	535‡	330
C	1	402	362
	2	470	410‡
	3	472	430‡
D	1	470	382‡

‡, differences in potency is higher than 20%

Micro–FIow Imaging

Subvisible particles, particularly in the range of 0.1–10 μm, remain an important quality attributes despite uncertainty around their potential immunogenicity risk[13]. As **Figure 9** shows, total particle concentration of subvisible particles in all biocopy products with sizes ranging from 1–55 μm was measured by microscopic particle count test using micro–flow imaging. Total particle concentration differed markedly between products with brand B being the highest and brand D the lowest. Such differences were also apparent between batches of brand B and C. Our results also determined a higher amount of silicon oil (up to 1.5–fold) than proteinaceous particles at size greater than 1 μm in all biocopy products except brand D. In all brands, the total content of particles greater than 10 μm and 25 μm per vial/syringe did not exceed the limit stated on Ph. Eur. "particulate contamination: sub-visible particles" *i.e.* 3000 particles per container and 300 particles per container.[14]

DISCUSSION

Current study shows that the risk of anti-r-HuEpo–associated PRCA is high in the treated Thai population. Based on this registry, estimated PRCA rates with r-HuEpo biocopy products were 1.7 case/1,000 patient–year exposure. This is more than 100 times higher than the risk of PRCA associated with the innovator epoetin products worldwide[4]. Of more than 20 r-HuEpo biocopy brands available on the Thai drug market, four brands have been identified as being involved with PRCA.

Higher HMW species were detected in two brands which may indicate higher aggregate–containing r-HuEpo. These aggregates are likely to comprise r-HuEpo and/or HSA-r-HuEpo; thereby suggesting that these brands pose a higher risk of aggregation–induced immune response. Aggregation, in particular, has been universally recognized as a crucial factor to trigger unwanted immunogenic response[15]. With r-HuEpo, it can induce the formation of neutralizing antibody against r-HuEpo in patients[16,17]. These two brands were associated with 6 of the 9 reported PRCA cases, implying a link between aggregation and immunogenicity.

In one other brand, we determined substantially higher amount of CHO HCP impurities. Such impurities can have immunogenic effects[18] and have been suggested to impact the stability of biologics by increasing tendency of aggregation and fragmentation[19]. One case of r-HuEpo–mediated PRCA associated with this brand was reported in this registry suggesting another link between HCP impurities and immunogenicity.

Another important finding is isoform variation observed between r-HuEpo brands and between batches of the same brands. While such variations are expected between brands, likely due to different manufacturing processes, variations in isoform pattern between batches of the same brand may be an indicative of improper control of the production process. Although it is still not completely clear, different isoforms may also contribute to the overall biological activities and toxicity of a protein drug as well as immunogenicity[20,21]. In addition to isoform, the presence of subvisible non-proteinaceous particles *i.e.* silicone oil droplets has also been linked to immunogenicity[22]. The high content of silicone oil droplets found in the majority of the brands analyzed may be adsorbed by protein molecules; thereby creating a vaccine–like effect[23].

Lastly, differences in the *in vitro* potency of some brands and more importantly, of some batches were quite striking compared to of Eprex®. Although this may suggest different potency than stated on the label, there are other factors that should also be considered, *e.g.* the use of different bioassays or standards than Eprex®. In any case, mislabeling may lead to either under- or overdosing patients resulting in unwanted clinical effects. In extreme cases, a high dose of r-HuEpo can lead to dangerously high hematocrit values[24].

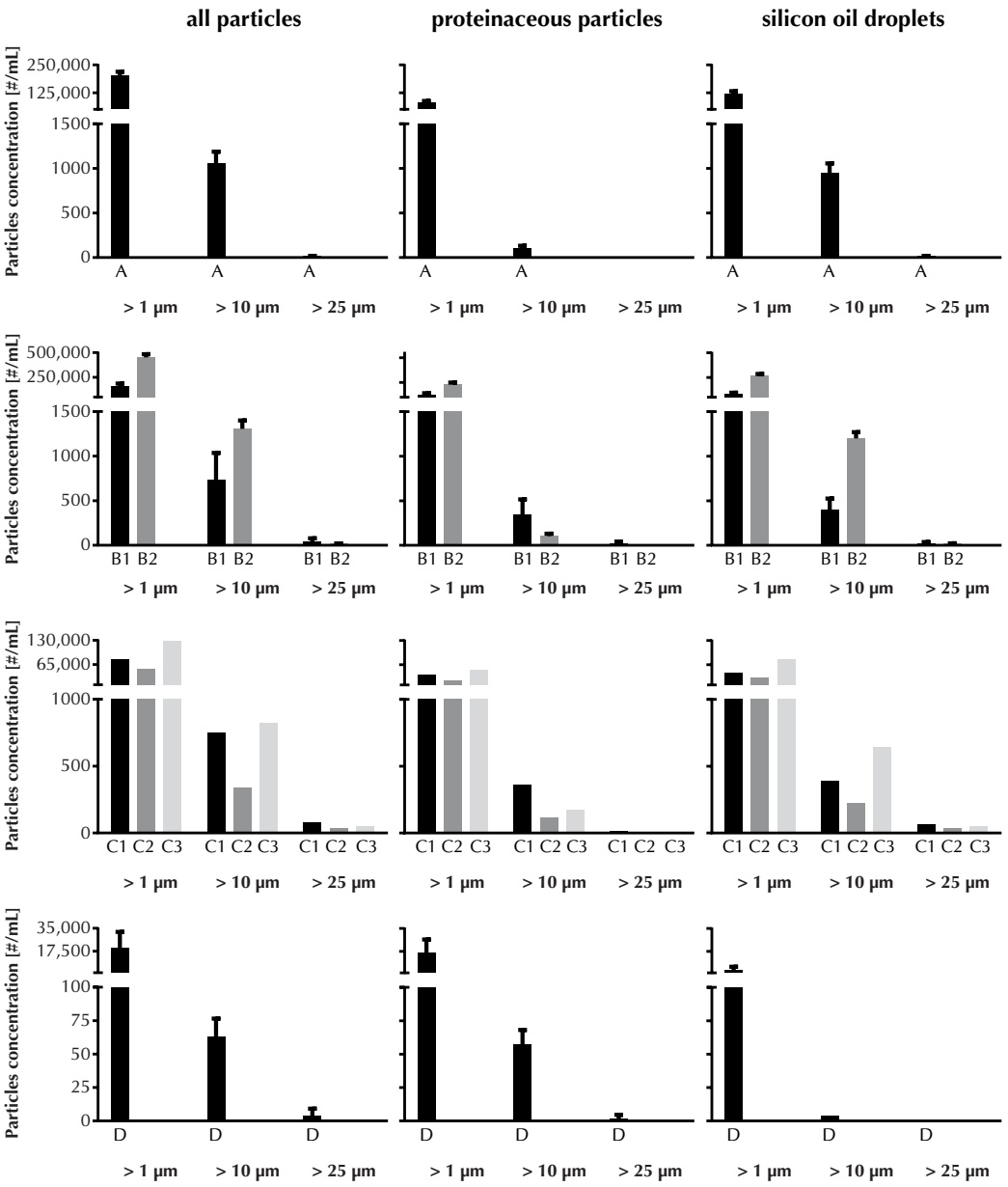

Figure 9. Particle counts for brand A (n=3), B(n=3), C(n=1) and D (n=2) presented in data bins on >1 µm, >10 µm and >25 µm.

Although we did not necessarily analyze the exact same batches of each r-HuEpo biocopy brands as the administered batches to the Thai patients, our observations show association between immunogenicity and process- and product-related impurities. The latter may arise during manufacturing and/or during handling and storage[7].

As is the case with other countries, copy biologics entered the Thai drug market before a biosimilar regulation pathway was in place. In addition, a full dossier is not requested and comparative clinical data is also not required. The Thai FDA, is currently in the process of re-evaluating all authorized biocopy products. With these data, we expect to further convince the regulatory body that despite being a resource limited country, there is a need to evaluate biocopy products using biosimilar guidelines *e.g.* full quality dossier including comparative data and to conduct pharmacovigilance as well as a risk management plan.

METHODS

Registry Design

This multicenter, immunogenicity surveillance registry employed a prospective cohort design, with enrollment of incident patients who were exposed to an r-HuEpo biocopy product by the SC route of administration, to estimate the incidence and prevalence of anti-r-HuEpo–associated PRCA. Each subject was observed for up to 3 years post-enrollment.

The registry did not provide any inducement to change therapy and was non-interventional. The treating physician was responsible for all decisions regarding the subject's clinical care and treatment. Subjects were enrolled through their healthcare providers for their clinical status. The incidence rate ratios were adjusted through stratification by duration of exposure. This registry has been approved by institutional ethical committee/institutional review board (IEC/IRB) of Faculty of Medicine Chulalongkorn University (IRB No 404/50) and Ministry of Health (Ref No 2/2551). The study was registered by ClinicalTrial.gov (NCT 00799019)[25].

Inclusion/Exclusion Criteria

The inclusion criteria included subjects who had been receiving (within 3 months) or about to receive (within 1 month) a marketed r-HuEpo product by the SC route of administration, were 18 years of age or older, were likely to continue to receive r-HuEpo product by the SC route for at least 1 year, read and signed the informed consent document for this registry indicating that they understood the purpose of and procedures required for the registry, and were willing to participate in the registry. The exclusion criteria included subjects who were unable to have adequate follow-up information, had a history of PRCA or aplastic anemia, had experience of unexplained loss or lack of effect (LOE) to a r-HuEpo product ongoing at the time of enrollment, had prior r-HuEpo treatment, had history of primary lack of efficacy, had history of r-HuEpo antibody prior to enrollment, were currently receiving immunosuppressive medications (*e.g.*, cyclosporine, tacrolimus, mTOR inhibitors, mycophenolic mofetil, azathioprine, or monoclonal antibodies) and prednisolone, or had previously participated in this registry.

Anti-r-HuEpo Antibody Assay

An anti-r-HuEpo antibody assay was tested as an investigation for unexplained LOE cases after enrollment. The assay was done at Division of Nephrology Laboratory, Chulalongkorn University by radio-immunoprecipitation assay. The serum samples were incubated with ^{125}iodine-labeled erythropoietin (Epo) purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). The bound antibody was separated from the free-labeled Epo using Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden). The beads were then thoroughly washed and counted on a gamma counter. The presence of anti-r-HuEpo antibody in the tested sera was determined by calculating the percent of total counts per minute (cpm). The serum analyzed in the screening assay was re-assayed if the percent of total cpm count was more than 0.3%. The titer assay was performed at dilutions of 1:20, 1:50, 1:100, 1:1,000, 1:10,000 and 1:20,000. The positive anti-r-HuEpo antibody was the sera with a total cpm count more than 0.9% at a 1:20 dilution of sera.

Statistical Methods

Incidence rate was estimated as the total number of cases of anti-r-HuEpo associated PRCA divided by the number of person–years of subcutaneous exposure. The exposure was classified as mixed when subjects switched across multiple products for a given quarter calendar year. Estimated glomerular filtration rate (eGFR) was calculated by Thai eGFR equation. The incidence rate ratio was adjusted for duration of exposure by stratification. Confidence intervals for rate estimates were calculated using the Poisson distribution for rare events.

Product Quality Analytics

R-HuEpo biocopy products identified to be involved with PRCA cases were characterized using different analytical methods, namely sodium dodecyl sulfate–polyacrylamide gel electrophoresis and iso–electric focusing, both in combination with western blotting, high–performance size–exclusion chromatography, endotoxin testing, Chinese hamster ovary host cell proteins impurities, micro–flow imaging, and *in vitro* bioassay.

R-HuEpo biocopy products were purchased from local pharmacies in Bangkok, Thailand, and shipped via a certified courier to Utrecht University, Utrecht, The Netherlands. Products were available at different strengths *i.e.* 2,000 IU/mL, 4,000 IU/mL and 10,000 IU/mL as well as different volumes *i.e.* 2 mL, 1 mL and 0.4 mL. Products were temperature monitored during shipping to ensure a proper cold chain. All analyses were performed before the expiry dates and one or more batches were assessed for batch-to-batch variability.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis in Combination with Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequent western blotting were performed under non-reducing and reducing conditions. Per r-HuEpo sample, an amount corresponding to 50 IU was mixed with 1x Bolt lithium dodecyl sulfate sample buffer with or without Bolt reducing agent containing 50 mM dithiothreitol (DTT) to a final volume of 40 µL.

All samples were subsequently heated at 70°C for 10 min and loaded on a Bolt 4–12% Bis-Tris Plus Gel. The PageRuler prestained protein ladder (10–180 kDa) was used as molecular weight standard. Separation was performed at a constant voltage of 200 V for 32 min.

After separation, proteins were transferred to an iBolt Transfer Stack, PVDF, 0.2 µm using the iBolt 7–Minute Blotting System following the manufacturer's recommendations. The blot was then incubated in blocking buffer (5% elk skimmed milk powder [Campina, Woerden, The Netherlands] in 1x TBS-$T_{0.05}$ (50 mM TRIS, 150 mM sodium chloride and 0.05% [w/v] Tween 20, adjusted to pH 7.4 with HCl) for 60 min at room temperature (RT) *i.e.* ±25°C.

Bands that correspond to r-HuEpo were detected with a primary antibody solution directed against human r-HuEpo at a working concentration of 4 µg/mL (custom-made rabbit polyclonal anti–r-HuEpo, Biogenes, Germany) in blocking buffer by overnight incubation at 4°C. Excess primary antibody was removed by rinsing the blot with 1x TBS-$T_{0.05}$ for 4 times each time for 5 min. Hereafter, the blot was incubated with anti-rabbit (goat polyclonal, horseradish peroxidase labeled, 1:10,000, Jackson Immuno Research, West Grove, Pennsylvania) for 60 min at RT followed by 4 times washing with 1x TBS-$T_{0.05}$ each time for 5 min. The blot was then exposed to Super Signal West Femto Maximum Sensitivity Substrate for 30s at room temperature. Bands were visualized with a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software version 4.6.9 (Bio-Rad, Hercules, California, USA). Incubation and washing steps were always performed with constant orbital shaking. Unless stated otherwise, all products used were obtained from Thermo Fisher Scientific, Bleiswijk, The Netherlands.

Iso–Electric Focusing in Combination with Western Blotting

To assess differences in isoform pattern between brands and between batches of the same brand, iso–electric focusing (IEF) and subsequent western blotting were performed. Samples were prepared following the manufacturer's recommendations. Per r-HuEpo sample, an amount corresponding to 50 IU was diluted in DeStreak rehydration solution containing 0.5% IPG buffer pH 4-7 and 20 mM DL-DTT (Merck, Darmstadt, Germany) to a final volume of 340 µL. The sample in rehydrated solution was transferred to each lane of IEF rehydration tray where the 18 cm Immobiline Dry Strip gels (IPG strips) pH 4-7 was placed onto the sample gel side down. The IPG strips were then overlaid with Immobiline cover fluid to prevent evaporation. The IPG strips were rehydrated overnight (11–16 hours) at RT where samples were passively taken up, after which the IPG strips–containing samples were transferred to the IPGphor coffins. Subsequently, the IPGphor coffins were placed on the platform of Ettan IPGphorII IEF system and covered with the supplied box. The IEF of the IPG strips was performed at a platform temperature of 20°C with constant current of 50 µA per strip at multiple steps as listed on **Table 8**.

IPG strips were either stored immediately at -80°C after the run or were fixed in fixing solution (Sigma-Aldrich), aligned, and blotted to one PVDF membrane (Bio-Rad) following Towbin's method (maximum 8 strips per blot)[26]. Blots were blocked, incubated and detected as described on previous section. Isoform bands across lanes were analyzed using Quantity One software as described in the user guide where the distance migrated by each isoform band from the top of the blot was relatively compared to total blot length and expressed in relative mobility (R_f). Hereafter, R_f was plotted against intensity of the band. Unless stated otherwise, all products used were

obtained from GE Healthcare, Eindhoven, The Netherlands).

Table 8. Protocol for First–Dimension IEF

Step	Voltage (Volt)	Volt–hours[†]	Volt gradient type
1[‡]	100	200	Step and Hold
2	300	150	Step and Hold
3	500	250	Step and Hold
4	1000	500	Step and Hold
5	8000	3000	Gradient
6	8000	20,000	Step and Hold
	Total	24,100	

[†], because the optimal time for focusing may vary, it is preferable to program a protocol based on volt–hours rather than on hour
[‡], new electrode strips were placed every 40 min

High–Performance Size–Exclusion Chromatography

The presence of soluble aggregates was assessed on an Agilent 1200 apparatus (Agilent Technologies, Palo Alto, California, USA) with a UV detector set at 214 nm and combined with a multiangle laser light scattering (MALLS) detector DAWN HELEOS (Wyatt Technology Europe GmbH, Dernbach, Germany). The mobile phase buffer was composed of 1.5 mM potassium phosphate, 8.1 mM sodium phosphate and 0.4 M sodium chloride at pH 7.4 and filtered through a 0.2 µm filter (Sigma-Aldrich, St. Louis, Missouri, USA) prior to use.

Prior to measurement, the r-HuEpo products were diluted to a concentration of 1,000 IU/mL using mobile phase buffer. Formulation buffers were prepared in accordance to the products' leaflets. In the case of brand C, formulation buffer was not prepared because none is stated in the products' leaflet. As a control, the erythropoietin for physicochemical tests CRS (the European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France) was dissolved in 100 µL citrate buffer and incubated at 55°C for 14 days as described in the Ph. Eur. Monograph for erythropoietin concentrated solution[27]. Subsequently, the preparation was further diluted to 0.2 mg/mL in mobile phase buffer. Fifty microliters of both r-HuEpo sample and control were loaded onto a TSKgel G3000SWxl (Tosoh Bioscience, Stuttgart, Germany) and separation was performed at room temperature with 0.5 mL/min. The root mean square (rms) diameter was calculated from the MALLS signal, using the Berry Fit option of the Astra software version 5.3.2.22 (Wyatt Technology Europe GmbH, Dernbach, Germany).

Endotoxin testing

Endotoxin levels in the r-HuEpo biocopy products were assessed with a colorimetric Limulus amebocyte Lysate (LAL) assay (Lonza, Base, Switzerland). All measurements were performed by Lonza (Verviers, Belgium) and samples were temperature–controlled transported to Lonza using a certified courier. The assay procedure was performed as follows. Firstly, samples were diluted ten times in LAL reagent water, followed by heat treatment at 75°C for 30 min to inactivate HSA.

Per product, a volume of 100 µL of a 1:100 dilution was dispensed onto a microplate well in duplicate. To verify that the products themselves would not interfere with endotoxin measurements, an additional plate was prepared using the same dilutions (duplicate) of each r-HuEpo copy product, and 0.5 endotoxin unit per milliliter (EU/mL) of *Escherichia coli* (*E. coli*) endotoxin stock solution was used to spike the samples. Plates (spiked and unspiked) were tapped gently on the side repeatedly to facilitate mixing followed by incubation for an additional 10 min. Absorbance was immediately read at 405–410 nm using a Lonza Kinetic QCL Reader BE25-315 with WinKQC software. Distilled water was used to adjust the photometer to zero absorbance. A standard curve with the best–fit straight line was generated using an *E. coli* endotoxin standard prepared at five different concentrations ranging from 0.005 to 50 EU/mL. The endotoxin concentration in each of the r-HuEpo samples was calculated based on the generated equation.

Chinese Hamster Ovary Host Cell Proteins Enzyme–Linked Immunosorbent Assay

To quantify traces of Chinese hamster ovary proteins in the r-HuEpo products, a CHO host cell protein (HCP) enzyme–linked immunosorbent assay, 3G (Cygnus Technology, Southport, North Carolina, USA) was used according to the manufacturer's recommendations. In short, r-HuEpo products were diluted at three different strengths, namely 1:2; 1:10 and 1:100, in sample diluent buffer to test for high dose hook effect. In addition, r-HuEpo products were spiked by diluting them in known concentration of HCP standard *e.g.* 100 ng/mL at the same dilution folds to determine whether anything in the sample interferes in accurately measuring HCP concentrations. Subsequently, 50 µL of the standards and diluted r-HuEpo products (unspiked and spiked) were transferred in duplicate onto a 96–well plate pre-coated with affinity purified capture goat polyclonal anti-CHO antibodies. Samples were incubated with horseradish peroxidase–labeled goat polyclonal anti-CHO antibodies for 2 h at room temperature with constant orbital shaking (180 rpm). The supernatant was discarded and the plate was washed four times with washing buffer (TRIS–buffered saline). Upon washing, plates were developed with 3,3′,5,5′–Tetramethylbenzidine substrate for 30 min without shaking. The development was stopped by adding 0.5 N sulfuric acid. Optical density was measured at 450 and 650 nm (reference wavelength) using SPECTRO$^{star\ Nano}$ (BMG LABTECH GmbH, Ortenberg, Germany). Values were corrected for background and a standard curve was constructed using the supplied CHO HCP standard. Data was fitted to a 4–parameter logistic fit using Prism 6 software version 6.07. Subsequently, HCP content in each r-HuEpo formulation was determined.

Micro–Flow Imaging

R-HuEpo copies were prepared and left at room temperature for at least 5 min prior to measurement to diminish any air bubbles. In each run, 500 µL of sample was drawn into 1,000 µL pipette tip and fixed into an inlet port of micro–flow imaging (MFI) DPA–5200 system (Protein Simple, Santa Clara, California, USA) equipped with a 100 µm flow cell, operated at high magnification (14x) and controlled by the MFI View Software version 6.9 was used. In between sample runs, a sequence of MQ-water, 2% Hellmanex (Hellma Analytics, Müllheim, Germany) and MQ-water was flushed through the system to provide a clean baseline. Subsequently, MQ-water was used to confirm

that the tubing was properly cleaned indicated by particles count of less than 500 particles/mL. Subsequently, "optimize illumination" was performed using MQ-water, as formulation buffer was not known for all r-HuEpo copies. Silicon oil droplets were discriminated from proteinaceous particles by applying filters Intensity Min and Aspect Ratio as described elsewhere, using MFI view application suite version 1.4.3[28]. Output data were formatted to greater than 1, 10 and 25 μm.

In Vitro Bioassay

Erythroid cultures were generated from bone marrow cells collected from bone marrow transplant donors with informed consent as previously described[29]. Hereafter, r-HuEpo biocopy products and Eprex® were prepared at different potency, namely 0.5 and 1.0 IU/mL final concentration and were added to the cultures in duplicate. The ability of different r-HuEpo biocopy products to induce the proliferation of erythroid progenitor cells was compared to Eprex®.

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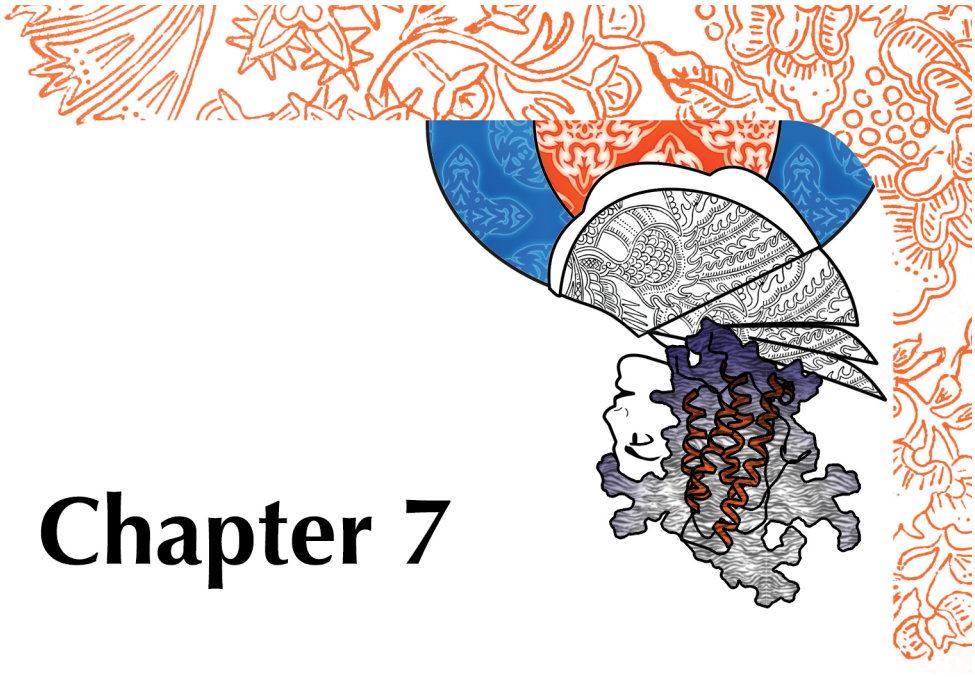

Chapter 7

Summary and Perspectives

Liem Andhyk Halim

SUMMARY

In the introduction (**Chapter 1**), we described biologics and their subsequent versions *i.e.* biosimilars (a term introduced by the EMA). Following the expiration of patent and exclusivity arrangements for innovator biologics, these so-called biosimilars recently have entered the market. Although biosimilars are seen as cost-effective alternatives to high-priced innovator biologics[1] in some regions their implementation in standard of care treatment is still low. This could be because it is sometimes unclear whether they are as safe and effective as the innovator product[2]. In addition, as reported elsewhere, a low awareness of biosimilars amongst patients may contribute to the low adoption rate for biosimilars[3].

Table 1. Characteristics of small molecule drugs compared to biologicals as adapted from [5].

		Chemical drugs	Biologics
Properties	Size	Small (single molecule), low molecular weight	Large (mixture of related molecules), high molecular weight
	Structure	Simple, well-defined, independent of manufacturing process	Complex (heterogeneous), defined by the exact manufacturing process
	Stability	Stable	Unstable, sensitive to external conditions
	Modifications	Well – defined	Many options, such as post-translational glycosylation
	Manufacturing	Predictable chemical process	Unique line of living cells Impossible to make identical copy
	Characterization	Identical copy can be made Easy to characterize completely	Impossible to characterize fully due to molecular composition and heterogeneity
	Immunogenicity	Mostly non-immunogenic	Immunogenic

Biologics differ from small molecule chemical drugs in terms of biochemical properties, manufacturing processes, product characterization and immunogenicity (**Table 1**). As a result, these are very complex drugs, and physicochemical analyses alone are not sensitive enough to predict whether the biological and clinical properties of biosimilars are comparable to the innovator. This highlights the need for an alternative regulatory framework as the current one for market approval of generic drugs is not suitable for granting the marketing authorization of biosimilars[4]. An alternative has been offered by the EMA, which pioneered the development of a biosimilar regulatory framework[6,7]. The EMA now regulates most biosimilar products worldwide[8].

At present, biosimilar–specific safety issues including immunogenicity have not been reported for any of the marketed biosimilars in the EU, although there is one instance in which a difference in potency is observed between a biosimilar and the innovator product[9]. This gives reassurance that the EU tailored biosimilar pathway is enabling the introduction of efficacious and safe products. Nonetheless, the biosimilar guidelines still rely on clinical data, meaning that quality differences on the product level are acceptable only as long as there are no clinical issues[10]. This is likely because there is still a knowledge gap between quality attribute of a biologic product and its clinical outcomes[11].

The studies presented in this thesis therefore aim at establishing a link between quality attributes of biosimilars and potential clinical outcomes regarding to safety and immunogenicity. The research involved linking comparative qualitative data of granulocyte colony-stimulating factor (G-CSF) and erythropoietin (epoetin) to clinical safety and immunogenicity.

Thus far, within the biosimilar guidelines a comparability exercise is intended to demonstrate that the quality, safety and efficacy of the biosimilar are comparable to the innovator; thereby this is always required by regulatory agencies. Such exercises, particularly at the clinical level, are costly and take time, thereby adding to the biosimilar's development costs. This is in contrary to what biosimilars are trying to achieve *i.e.* a cheaper alternative[14]. Not only has it been pointed out that conducting extra clinical studies is unnecessary and unethical[15], the added value of clinical comparability studies has also been questioned by some experts[10]. They believe that current biosimilar comparability exercise could hinder the development of the next wave of biosimilars *e.g.* monoclonal antibodies (mAbs). Despite this, a number of medical societies demand more and longer clinical studies in order to demonstrate similar safety and efficacy profiles for biosimilars, because they are still not convinced of their comparability with the innovator products[12,13]. To contribute to the debate regarding the added value of biosimilar comparability exercises beyond quality**,** we have reviewed the evidence base for the unpredictability of safety issues of biologics In **Chapter 2**.

There are only two issues with biosimilars: potency and immunogenicity. Although predicting immunogenicity of biologics remains elusive, similarity in immunogenicity between a biosimilar and its innovator product is predictable. Differences in intrinsic immunogenicity of the biosimilar are not expected, because a biosimilar is expected to share the amino acid sequence and structure of the innovator. Differences in immunogenicity can only be expected if a difference in impurities is identified. Thus, if no differences in impurity profiles between biosimilar and innovator can be found, differences in immunogenicity are very unlikely. Other supporting arguments that the degree of similarity in immunogenicity between biosimilars and the innovator can be predicted from quality data are the legion of manufacturing changes of marketed biologics done with no examples of unexpected immunogenicity. Further, current biosimilars have been as safe and efficacious as the innovator despites some differences in physiochemical and biological characteristics. Other safety issues associated with biologics include pharmacodynamic effects, thereby related to potency. Only if the potency of a biosimilar differs from the innovator will it fail to obtain comparable efficacy and safety profiles. All in all, this finding implies that there is nothing else needed for biosimilar comparability exercise beyond product quality, and that quality comparability exercise by itself is sufficient.

In addition to biosimilars, so-called copy biologics are available in less regulated markets such as parts of South America and Asia. There is little data regarding direct comparison between a biosimilar and a copy biologic and an innovator product. Copy biologics have not been approved through stringent regulatory process and therefore, are often perceived by clinicians as being inferior to biosimilars[16]. To investigate their value, we described in **Chapter 3** a head-to-head comparative quality study of biosimilar and copy products of one form of G-CSF *i.e.* filgrastim. Analytical and biological assays which include (1) high–performance size–exclusion chromatography (HP–SEC), (2) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), (3) reversed–phase

chromatography and (4) endotoxin contamination as well as (5) *in vitro* potency assay were used. We demonstrated that biosimilar filgrastim products, Zarzio® and Tevagrastim®, have comparable quality to the innovator product, Neupogen®. The results also indicated that Biocilin® showed a significantly lower and PDgrastim® a higher specific activity compared to the biosimilar and the innovator filgrastim products. Other than these, no clinically significant differences between the filgrastim products were found.

Another comparative quality study is described in **Chapter 4** for two EU–approved biosimilar epoetin products (Retacrit® and Binocrit®). These were compared to their innovator products epoetin alpha (Eprex®). In addition, we included quality assessment of another innovator product epoetin beta (NeoRecormon®) as we had access to this product. This study is a continuation of previous publication by our group with addition of multiple lots of each products[17]. Results showed that all products analyzed contained neither higher molecular weight (HMW) species nor fragments as revealed by SDS–PAGE. Correspondingly, HP–SEC and asymmetrical flow field–flow fractionation (AF4) detected no soluble aggregates in these four epoetin products. At the same time, epoetin monomer content was found to differ between products and between batches of a single product. Owing to variation in purification processes and due to a difference in the host cells used to produce the protein, isoform variation between epoetin products was also apparent as indicated by capillary zone electrophoresis analysis. Such variation was not observed between batches of all products. This consistency between batches demonstrates a well-controlled manufacturing process. Likewise, differences in the ratio of *in vitro* and *in vivo* potency between tested epoetins were observed but, not surprisingly, were less striking when comparing different batches of the same product. Such differences are likely due to varying degrees of sialylation induced by different manufacturing processes.

Despites differences in quality attributes found in our study, no clinical safety concerns have emerged associated with biosimilar epoetin products in the EU. In other parts of the world where copy biologics came onto the market before biosimilar regulations were in place, physicians did find safety issues *e.g.* an increased incidence of pure red cell aplasia (PRCA) in Thailand. Such incidents coincided with the approval of more than 20 copies of epoetin approved through the classical generic regulatory pathway in Thailand. Various factors have been suggested as the cause of such incidents, including genetic background[18], handling and storage[19] as well as product quality attributes. As the latter had not been previously studied, **Chapter 5** describes a study in which we compared the quality of 12 different copy epoetin alpha products to the innovator epoetin alpha product, Epogen®. All copies used human serum albumin (HSA) as excipient although this no longer complies with EU regulations. Our results showed high aggregate epoetin/HSA levels and a substantial amount of protein fragments detected in some copies using SDS–PAGE, HP–SEC and AF4. In one copy epoetin product, high endotoxin levels were also detected. With the use of these copy epoetin products, there is an increasing trend of PRCA among Thai patients. This strongly supports a link between the quality attributes of these copies and their immunogenicity.

In the next chapter of this thesis we investigated further the link between quality data and clinical outcomes. Both a prospective pharmacovigilance study on Thai patients receiving copy epoetin products and an analytical study looking at product qualities of these products were initiated. The data presented in **Chapter 6** on 4,018 patients enrolled in the registry showed that

9 patients were tested positive for the formation of anti-epoetin neutralizing antibodies. These 9 PRCA cases were associated with 4 of the 15 copy epoetin brands in the registry. As with the studies described in chapter 5, these four brands were compared and a significant variation in physicochemical characteristics was found. SDS–PAGE in combination with western blotting showed traces of epoetin at HMW ranges in brand A and B. Similarly, higher % of HMW content was also determined in these two brands. Meanwhile, the amount of host cell protein impurities in brand D was determined to be higher than the typical purity target (100 ng/ml). With the exception of brand A, *in vitro* potency of all brands was higher than of Eprex®. In addition, isoform variations were apparent between analyzed brands as well as between batches of brand B and C. With the exception of brand D, all brands contained more non–proteinaceous subvisible particles, *i.e.* silicone oil, than proteinaceous particles. In sum, both analytical and clinical data suggest a potential link between quality attributes of copy epoetin products and the increasing incidence of PRCA in Thailand.

PERSPECTIVES

Biologics are complex mixtures of large and intricate molecules, making an identical copy a challenging task and the generic regulatory pathway not suitable to authorize copy biologics. In 2006 the EMA introduced the term biosimilars and was the first agency to introduce regulations for their approval. At present many countries have introduced biosimilar regulations with the EMA approach as model. In developing countries, many copies of proteins were marketed predating specific biosimilar regulations. For example, as discussed in this thesis, epoetin copy products were licensed in Thailand under the generic paradigm without having to submit a full quality dossier and comparative clinical data, which the biosimilar regulations now require.

More efforts are still needed to develop good biosimilar regulations adapted to local needs and demands and even more with the enforcement of the regulations. For example, the Thai FDA is currently re-evaluating copy biologic products currently in their market. Despite our data suggesting possible product impurities in some of the copy epoetin products, no license have yet been withdrawn for any of these products.

Indeed, our results show that while the quality of biosimilar epoetin products is high, some copy epoetin products available in the Thai drug market differ considerably from the innovator. These results do not disqualify all copy biologics produced in developing countries. Instead, they indicate that reliable research data regarding direct quality comparisons between copy and innovator products has been either lacking or the published data show only that a few copies lack quality comparability[19-22]. In view of this, manufacturers of copy biologics need to do comparisons between their products and the innovator and publish the data, as is the case with most biosimilar products approved in the EU.

Also, there is a need for more quantitative data to show biosimilarity. For instance, we have recently compared mass spectrophotometer spectra of three commercial EU-licensed epoetin products in which we define a biosimilarity score to quantitatively assess structural similarity. The details of this study will be published elsewhere. We also demonstrated the possibilities for accurately quantifying relative sialylation level of different epoetin products using multiplex surface plasmon resonance[23].

In addition, the EMA is putting more and more emphasis on comparative quality data to show similarity. Recently the EMA released a concept paper to revise the clinical requirements for granulocyte colony-stimulating factor (G-CSF) trying to establish criteria to waive clinical studies[24]. In conclusion, in this thesis quality data of biosimilars are linked to clinical outcome contributing to a future in which the biosimilars will be increasingly considered as classical generics.

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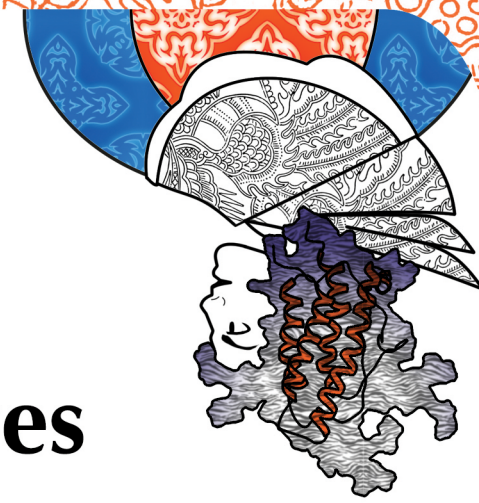

Appendices

Nederlandse samenvatting

List of abbreviations

List of publications

List of co–authors

Acknowledgments

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NEDERLANDSE SAMENVATTING

In de inleiding (**Hoofdstuk 1**) worden biologische geneesmiddelen, biologicals, en de nagemaakte versies, biosimilars (een term geïntroduceerd door de EMA), beschreven. Biosimilars mogen op de markt worden gebracht, als het octrooi en de exclusieve exploitatierechten van een biological zijn verstreken. Hoewel biosimilars worden gezien als een kosteneffectief alternatief voor de dure biologische geneesmiddelen, is de implementatie van biosimilars als zorgstandaard in bepaalde regio's van de wereld nog steeds laag. Het probleem is dat een biological door zijn oorsprong nooit exact te kopiëren is. Biosimilars zijn soortgelijk, maar niet identiek. Of biosimilars net zo veilig en effectief zijn als de originele biologicals zijn onduidelijk. Bovendien kan de geringe bekendheid van biosimilars bij patiënten bijdragen aan de lage in gebruik name van biosimilars.

Table 1. Kenmerken van traditionele geneesmiddelen en biologische geneesmiddelen, biologicals.

		Traditionele geneesmid-delen	Biologicals
Eigenschappen	Grootte	Klein (een enkel molecuul) Laag molecuulgewicht	Groot (mix van vergelijkbare moleculen) Hoog molecuulgewicht
	Structuur	Simpel, gedefinieerd, onafhankelijk van productieprocessen	Complex (heterogeen), gedefinieerd door de productieprocessen
	Stabiliteit	Stabiel	Instabiel, gevoelig voor externe factoren
	Modificaties	Goed gedefinieerd	Veel opties, zoals post-translationele glycosylering
	Productie	Voorspelbare uitkomst van chemische processen	Unieke cellijn
		Identieke kopie kan worden gesynthetiseerd	Onmogelijk om identieke kopieën te produceren
	Karakterisatie	Gemakkelijk en volledig te karakteriseren	Karakterisatie is onmogelijk door de moleculaire compositie en heterogeniteit
	Immunogenic-iteit	Vrijwel nooit immunogen	Immunogeen

Kleine moleculen en biologische geneesmiddelen verschillen van elkaar op het gebied van biochemische eigenschappen, productieprocessen, karakterisatie en immunogeniciteit (**Tabel 1**). Biologicals zijn zeer complexe geneesmiddelen en fysisch-chemische analyses zijn niet afdoende om te voorspellen of de biologische en klinische eigenschappen van biosimilars vergelijkbaar zijn met die van de originele biologicals. Dit benadrukt de noodzaak voor een alternatief regelgevingskader voor het verlenen van marketing authorisatie van biosimilars tegenover de huidige richtlijnen voor de goedkeuring van generieke geneesmiddelen. EMA heeft een alternatief geboden met de ontwikkeling van een biosimilar regelgevingskader. De EMA reguleert momenteel het merendeel van biosimilar producten wereldwijd.

Momenteel zijn er nog geen specifieke veiligheidsaspecten vastgesteld voor één van de geautoriseerde biosimilars in de EU. Wel is er één geval bekend, waarbij een verschil in potentie is waargenomen tussen het originele biologische geneesmiddel en de biosimilar. Dit geeft enige geruststelling dat de EU met dit biosimilar regelgevingskader effectieve en veilige biosimilars op de

markt introduceert. Desondanks baseren deze richtlijnen zich nog steeds op klinische gegevens, wat betekent dat kwaliteitsverschillen op productniveau alleen aanvaardbaar zijn zolang er zich geen klinische problemen voordoen. Dit komt waarschijnlijk doordat er een gebrek aan informatie is tussen de kwaliteit van een biologisch product en de uiteindelijk daarbij behorende klinische resultaten.

Dit proefschrift bundelt studies met als doel het verband te leggen tussen de kwaliteitskenmerken van biosimilars en de mogelijke klinische uitkomsten met betrekking tot veiligheid en immunogeniciteit. De studies koppelen derhalve kwalitatieve data van granulocyt kolonie–stimulerende factoren (G-CSF) en erytropoëtine (epoëtine) met klinische uitkomsten met betrekking tot veiligheid en immunogeniciteit.

Binnen de biosimilar richtlijnen wordt een vergelijkingsstudie geacht aan te tonen dat de kwaliteit, veiligheid en efficiëntie van de biosimilar en het originele product gelijk zijn. Dit wordt verplicht gesteld door de regelgevende agentschappen. Dergekijke studies zijn vooral op het klinische vlak duur en rijdrovend, waardoor de ontwikkelingskosten van de biosimilars toenemen. Dit is tegenstrijdig met wat er met biosimilars beoogd wordt, namelijk het bieden van een goedkoper alternatief. Naast dat erop gewezen werd dat het uitvoeren van extra klinische studies onnodig en onethisch kan zijn[15], werd er door sommige experts ook vraagtekens geplaatst bij de toegevoegde waarde van klinische vergelijkingsstudies. Ze geloven dat de huidige biosimilar vergelijkingsstudie de ontwikkeling van de volgende golf van biosimilars, bijv. monoclonale antilichamen (mAbs), tegen kunnen houden. Desondanks eisen een aantal medische organisaties meer en langere klinische studies om de vergelijkbare veiligheid en werkzaamheid van de biosimilars aan te tonen, omdat ze nog steeds niet overtuigd zijn van de vergelijkbaarheid met het originele product. Om bij te dragen aan het debat over de toegevoegde waarde van de biosimilar vergelijkingsstudie naast de kwaliteit, hebben we in **Hoofdstuk 2** het bewijs bestudeerd van de mate van voorspelbaarheid in de veiligheidsissues in relatie tot de klinische effecten van de biologics.

Er zijn slechts twee problematieken met betrekking tot biosimilars: potentie en immunogeniciteit. Hoewel het voorspellen van immunogeniciteit van biologicals onmogelijk blijft, zijn de gelijkenissen in immunogeniciteit tussen een biosimilar en de originele biological voorspelbaar. Verschillen in intrinsieke immunogeniciteit van biosimilars worden niet verwacht, aangezien de aminozuursequentie en structuur van biosimilar en de originele biological overeenkomen. Verschillen in immunogeniciteit wordt alleen verwacht, wanneer divergenties in verontreinigingen worden aangetoond. Het is zeer onwaarschijnlijk dat immunogeniciteit verschilt, wanneer eenzelfde zuiverheidsprofiel wordt bepaald. Andere ondersteunende argumenten dat de verschillen in de vergelijkbaarheid van de immunogeniciteit tussen de biosimilar en het originele product voorspeld kan worden uit de kwaliteit van de data, komt voort uit de grote hoeveelheid aan veranderingen die zijn aangebracht in het productieproces van de biologics die op de markt zijn, waarbij geen enkele voorbeelden van onverwachte immunogeniciteit op zijn getreden. Daarnaast zijn de huidige, geautoriseerde biosimilars, ondanks de kleine verschillen in fysisch en biologische kenmerken, even veilig en werkzaam als de originele biologicals. Andere veiligheidskwesties, die in verband worden gebracht met biologicals, omvatten farmacodynamische effecten, dus gerelateerd aan potentie. Als de potentie van een biosimilar verschilt van de originele biological, kan er geen vergelijkbare werkzaamheid en veiligheidsprofiel worden verkregen. Al met al impliceren deze

bevindingen dat kwaliteitscontroles voldoende zijn. Er zijn geen aanvullende studies nodig om de vergelijkbaarheid tussen biological en biosimilar te beoordelen.

In minder gereguleerde markten, bijvoorbeeld delen van Zuid-Amerika en Azië, zijn naast de biosimilars zogenaamde biological kopieën beschikbaar. Er is weinig informatie voorhanden, waarbij een directe vergelijking wordt gemaakt tussen de originele biologicals, de kopie en de biosimilar. Biological kopieën worden niet geautoriseerd voor marketing door de aanwezigheid van stringente regelgeving. Clinici beschouwen deze kopieën dan ook als inferieur aan biosimilars[16]. In **Hoofdstuk 3** wordt een vergelijkend kwaliteitsstudie tussen biosimilar en biological kopieën van één G-CSF analoog, filgrastim, beschreven. Analytische en biologische assays, zoals (1) high–performance size–exclusion chromatografie (HP–SEC), (2) natriumdodecylsulfaat polyacrylamide gelelektroforese (SDS–PAGE), (3) omgekeerde fase chromatografie en (4) endotoxine detectie en (5) in vitro potentie assays werden toegepast. We hebben aangetoond dat biosimilar filgrastim producten, Zarzio® en Tevagrastim® kwalitatief overeen komen met het originele biological product, Neupogen®. De resultaten toonden wel aan dat Biocilin® een significant lagere activiteit heeft en dat PDgrastim® een hogere specifieke activiteit heeft in vergelijking met de biosimilars en de originele biological producten van filgrastim. Verder werden er geen klinisch relevante verschillen gevonden.

Een andere vergelijkend kwaliteitsonderzoek wordt beschreven in **Hoofdstuk 4**. In dit hoofdstuk worden twee EU goedgekeurde biosimilar epoëtineproducten (Retacrit® en Binocrit®) vergeleken met de originele biological, epoëtine alfa (Eprex®). Daarnaast is ook de kwaliteit van een andere biological, epoëtine beta (NeoRecormon®) beoordeeld. Dit onderzoek is een vervolg op een eerdere publicatie van onze groep[17]. Door middel van SDS–PAGE is aangetoond dat alle geanalyseerde producten geen componenten van hoger moleculair gewicht (HMW) of fragmenten bevatten. HP–SEC en asymmetrical flow field–flow fractionering (AF4) hebben geen oplosbaar aggregaten gedetecteerd in deze vier epoëtine producten. Er werden wel verschillen in epoëtine monomeergehalte aangetoond tussen de verschillende producten en tussen batches van een enkel product. Door de variaties in de zuiveringsprocessen en variaties in het gebruik van gastheercellen voor de productie van het eiwit, zagen we ook isovorm variatie tussen epoëtineproducten door middel van capillaire elektroforese analyse. Deze variatie werd niet waargenomen tussen batches van alle producten. Hieruit blijkt dat de productieprocessen binnen een enkel product gecontroleerd verlopen. Voor alle producten zijn ook de in vitro en in vivo potentie getest. Er zijn variaties in potentie tussen de verschillende producten waargenomen, maar deze waren minder opvallend tegenover de variaties tussen batches van hetzelfde product. Dergelijke verschillen zijn waarschijnlijk te wijten aan de mate van sialylering, die worden geïnduceerd door verschillende productieprocessen.

Ondanks dat er verschillen in kwaliteitskenmerken zijn waargenomen in ons onderzoek, zijn er geen klinische veiligheidskwesties in verband gebracht met epoëtine biosimilar producten in de EU. In andere delen van de wereld, waar de biological kopieën op de markt kwamen voordat de biosimilar regelgeving van kracht was, zijn wel veiligheidsproblemen waargenomen door artsen: bijvoorbeeld een verhoogde incidentie van rode bloedcel aplasie (PRCA) in Thailand. In Thailand vielen dergelijke incidenten samen met de goedkeuring van meer dan 20 verschillende epoëtine kopieën door de toepassing van de klassieke generieke regelgevingskader. Verschillende factoren

worden als mogelijke oorzaak van dergelijke incidenten bestempeld: genetische achtergrond, producthantering en opslag, en de kwaliteitskenmerken van het product. Omdat laatstgenoemde voorheen nog niet bestudeerd is, beschrijft **Hoofdstuk 5** een studie waarin de kwaliteit van de 12 verschillende epoëtine alpha kopieën worden vergeleken met het originele epoëtine alfa product, Epogen®. Alle producten bevatten humaan serumalbumine (HSA) als hulpstof, hoewel producten met HSA niet langer volstaan binnen de EU-regelgeving. Onze resultaten toonden aan dat enkele epoetine kopieën hoge concentraties van epoëtine en HSA aggregaten bevatten. Daarnaast werd er ook aanzienlijke hoeveelheden eiwitfragmenten gedetecteerd met behulp van SDS–PAGE, HP–SEC en AF4. In één epoëtine kopie product, werden zelfs hoge concentraties endotoxine gemeten. In Thailand hangt de toename van patiënten met PRCA samen met toename in het gebruik van epoëtine kopieën. Deze bevindingen onderschrijven een verband tussen de kwaliteitskenmerken van deze kopieën en de immunogeniciteit.

In het volgende hoofdstuk van dit proefschrift wordt verder ingegaan op het verband tussen de kwaliteitskenmerken en de klinische resultaten. Zowel een studie over de veiligheid van verschillende epoëtine kopieën bij Thaise patients, als een analytische studie naar de kwaliteitskenmerken van deze producten werd geïnitieerd. **In Hoofdstuk 6** wordt data van 4018 patiënten die deelnamen aan het onderzoek gepresenteerd, waarbij 9 patiënten positief werden getest op de aanwezigheid van anti-epoëtine neutraliserende antilichamen. Vier van de 15 epoëtine merken werden gelinkt aan deze 9 patienten met PRCA. Vergelijkbaar met de beschreven studie in Hoofdstuk 5, werden deze vier merken met elkaar en met de originele biological vergeleken. Er werden aanzienlijke variaties in fysisch-chemische eigenschappen geïdentificeerd. SDS–PAGE in combinatie met western blotting toonden sporen van epoëtine at HMW reeks bij merkproducten A en B. Door middel van HP–SEC is aangetoond dat bij hetzelfde merkproducten componenten van hoger moleculair gewicht (HMW) bevatten. In merkproduct D was de hoeveelheid gastheercel verontreinigingen hoger te zijn dan de zuiverheidsnorm (100 ng/ml). Met uitzondering van het merk A, was de in vitro potentie van alle merkproducten hoger dan van Eprex®. Bovendien waren isovorm verschillen tussen de geanalyseerd merken en tussen merken B en C duidelijk aanwezig. Alle merken (met uitzondering van merk D) bevatten meer niet–eiwitachtige deeltjes, zoals siliconenolie. Kortom, zowel analytische en klinische gegevens suggereren dat er een mogelijke verband is tussen de kwaliteitskenmerken van de epoëtine kopieën en de toename in PRCA incidentie in Thailand.

LIST OF ABBREVIATIONS

ADA	anti–drug antibodies
AF4	asymmetrical flow field-flow fractionation
AIDS	acquired immune deficiency syndrome
CHO	chinese hamster ovary
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
EDQM	european directorate for the quality of medicines & healthcare
EPO-BRP	erythropoietin–biological reference preparations
EPO-CRS	erythropoietin–chemical reference substances
epoetin	erythropoetin
ELISA	enzyme–linked immunosorbent assay
EMA	european medicines agency
EU	endotoxin unit
FDA	food and drug administration
FLR	fluorescence
HMW	high-molecular weight
HP-SEC	high–performance size–exclusion chromatography
HSA	human serum albumin
IEF	iso-electric focusing
IS	internatioal standard
IU	international unit
kDa	kilo dalton
LAL	limulus amebocyte lysate
LMW	low molecular weight
MALLS	multiangle light scattering
MFI	micro–flow imaging
MU	million unit
ND	not determined
NIBSC	national institute for biological standards and control
PEG	polyethylene glycol
PFS	pre–filled syringe
Ph. Eur.	european pharmacopeia
PRCA	pure red cell aplasia
PS80	polysorbate 80
rhG-CSF	recombinant human granulocyte colony–stimulating factor
RP-UPLC	reversed-phase ultra performance liquid chromatography
RT	room temperature
R-AUC	relative–area under the curve

RHEPO, R-HuEpo	recombinant human erythropoietin
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
UV	ultraviolet
WHO	world health organization

LIST OF PUBLICATIONS

Related to this thesis

Halim LA, Márquez M, Maas-Bakker RF, Castañeda-Hernández G, Schellekens H. Quality Comparison of Biosimilar and Copy Biologic Filgrastim Products with the Innovator Product. Submitted for publication (2016).

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Andhyk

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Liem Andhyk Halim was born on June 12th, 1986 in Malang, Indonesia. After finishing his secondary education, he was admitted to HAN University of Applied Sciences, Nijmegen, The Netherlands in 2004 and was awarded with scholarship. He performed his Bachelor's research project at Philips Research, Eindhoven, The Netherlands and at École polytechnique fédérale de Lausanne, Switzerland. After graduation, he continued pursuing a master degree in Life Science and Technology at both Delft University of Technology and Leiden University. During his 2–year joint Master's programme, he did a 6-months internship at Merck Sharp & Dohme, Oss, The Netherlands and Master's research project at Leiden University Medical Center, The Netherlands. After graduation, he started his academic career in 2011 at Utrecht Institute for Pharmaceutical Sciences (UIPS) at the Department of Pharmaceutics at Utrecht University, the Netherlands, as a research assistant for 9 months. He then accepted a fully funded PhD offer at UIPS under the supervision of Prof. dr. Huub Schellekens and Dr. Vera Brinks. Andhyk focused his research on assessing the quality of biosimilars or copy biologics particularty epoetin and filgrastim products and trying to link these quality data to clinical outcomes. The results of this work are described in his PhD thesis entitled "Biosimilars–linking quality data to clinical outcomes". Currently, he is working as Quality Manager at Centre of Excellence for Affordable Biotherapeutics in Utrecht, The Netherlands.