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

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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.

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 microheterogeneity 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 2 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

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account of the use of different CHO cells strain.[13] 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.

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 5× 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 a 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 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) holotransferrin, 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]

Table 1
List of All Epoetin Products

Brand Name (INN)	Lot Number	Declared Potency	Excipients
Eprex (epoetin alfa)	DDS5L00 DGS4W00 DHS5T00 DIS3M00	4000 IU/0.4 mL	Sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, sodium chloride, glycine, polysorbate 80
Binocrit (epoetin alfa)	450112 730412 341211	10,000 IU/1.0 mL 8000 IU/0.8 mL	Sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, sodium chloride, glycine, polysorbate 80
Retacrit (epoetin zeta)	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

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 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.

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 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 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 detectorset at 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 erythroleukemia.[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 subcultured every 2-3 days and split 1:5 when they had reached a cell density of 2-5 × 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 × 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, PerkinElmer, 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 (PerkinElmer, Waltham, MA). Bioactivity estimates of the different preparations were derived relative to the epoetin standard (Third WHO International Standard [third WHO IS] for

Table 2
AF4 Elution Program Settings

Step	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

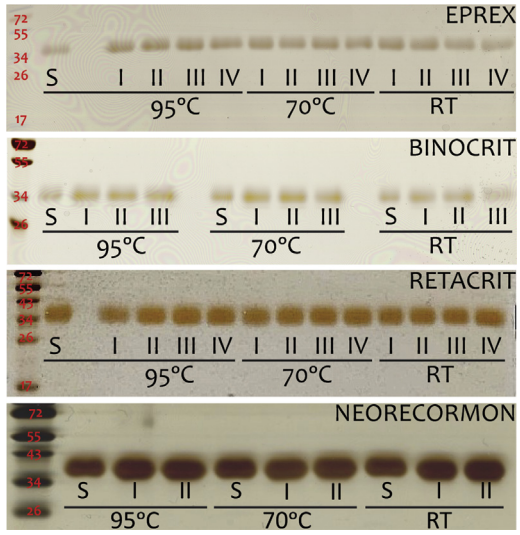

Figure 1. SDS-PAGE of all epoetin products under nonreducing condition. Roman number represents the different batches of each products. S is epoetin-BRP, and RT stands for room temperature. Different intensities are most likely due to gel-to-gel variation.

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. monograph 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 nonlinearity 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 (25°C), 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

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.

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

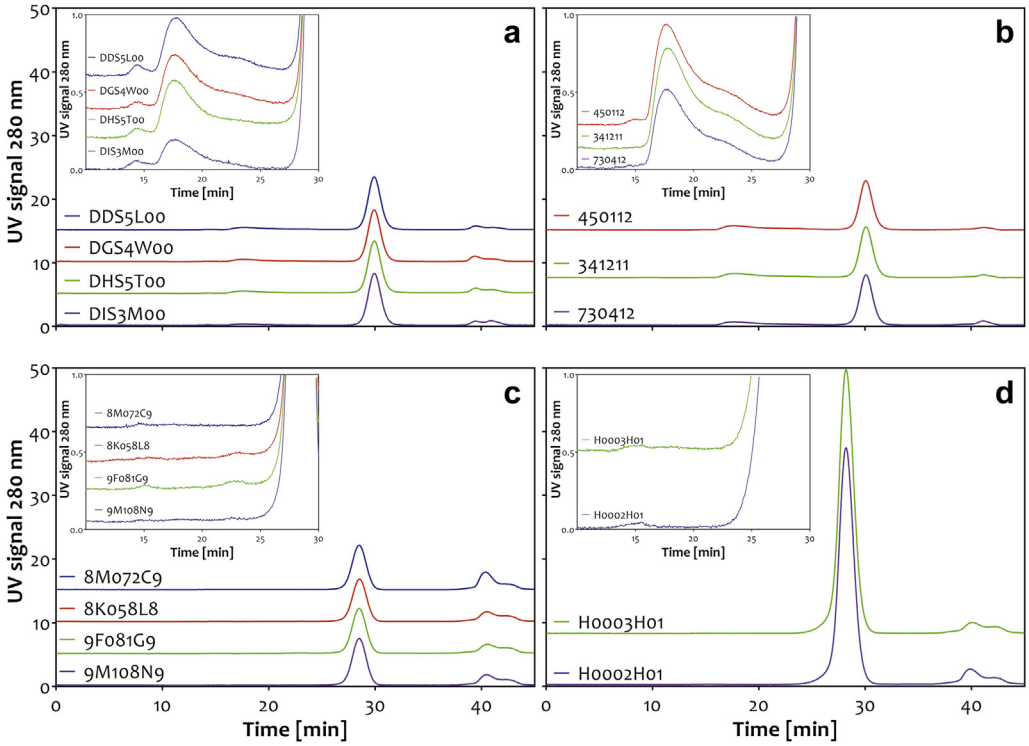

Figure 2. HP-SEC chromatograms of (a) Eprex, (b) Binocrit, (c) Retacrit, and (d) NeoRecormon. The inset is a zoom of the chromatograms of the area between 15 and 30 min. The different colors represent the batches of single product.

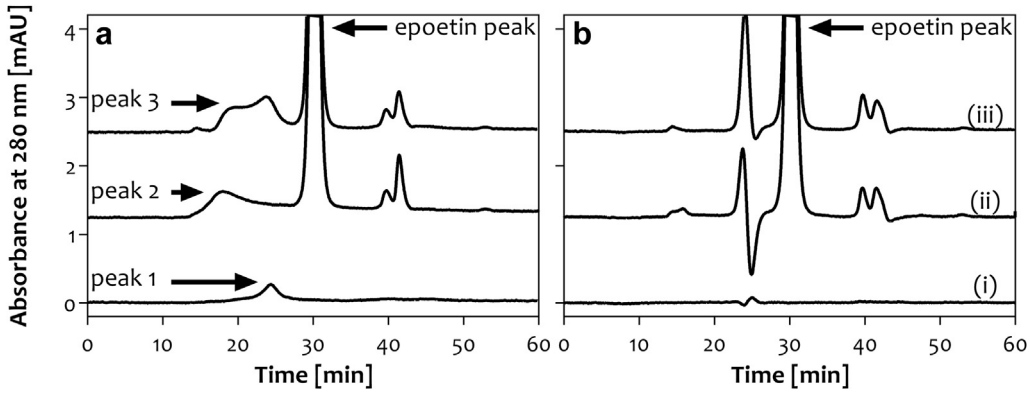

Figure 3. Zoomed HP-SEC chromatograms of (i) PS80, (ii) Eprex, and (iii) Eprex spiked with 0.3 mg/mL PS80 in (a) absence or (b) presence of 0.3 mg/mL PS80 in the mobile phase.

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.

To study whether the high–molecular weight (HMW) peak was due to PS80 or epoetin oligomers or both, (1) 0.3 mg/mL of PS80, (2) Eprex, and (3) Eprex spiked with 0.3 mg/mL of 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 of 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.

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.

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%).

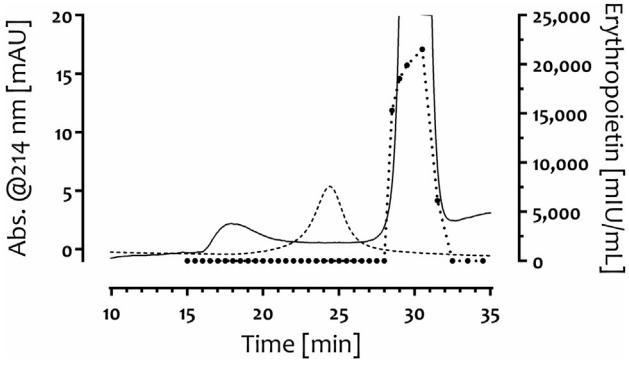

Figure 4. Chromatograms of Eprex batch DIS3M00 (solid line) and 0.3 mg/mL of PS80 (dashed line) on Superdex 200 column (left *y*-axis) and results from epoetin specific enzyme-linked immunosorbent assay (dotted line) on Eprex column fractions (right *y*-axis).

Asymmetrical Flow Field–Flow Fractionation

Asymmetrical flow field–flow fractionation (AF4) was used as an orthogonal method to HP-SEC for the separation and

Table 3
Comparison of Content of the 4 Epoetin Products Tested

Brand Name	Lot Number	Declared Content (IU/mL)	Content UV280 (IU/mL)	
			HP-SEC	AF4
Eprex	DDS5L00	10,000	9963 ± 2	9747 ± 38
	DGS4W00		9770 ± 35	9480 ± 189
	DHS5T00		9875 ± 120	9614 ± 76
	DIS3M00		9825 ± 100	9587 ± 38
	Mean		9858 ± 80	9607 ± 105
Retacrit	8K058L8	10,000	8014 ± 63	9275 ± 636
	8M072C9		8496 ± 52	10,059 ± 700
	9F081G9		8480 ± 81	9468 ± 369
	9M108N9		8808 ± 44	10,450 ± 587
	Mean		8450 ± 62	9812 ± 586
Binocrit	730412	10,000	9553 ± 7	11,008 ± 742
	4501121		9400 ± N.D.	10,185 ± 591
	341211		9803 ± 34	10,817 ± 265
	Mean		10,767 ± 27	11,940 ± 636
NeoRecormon	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.

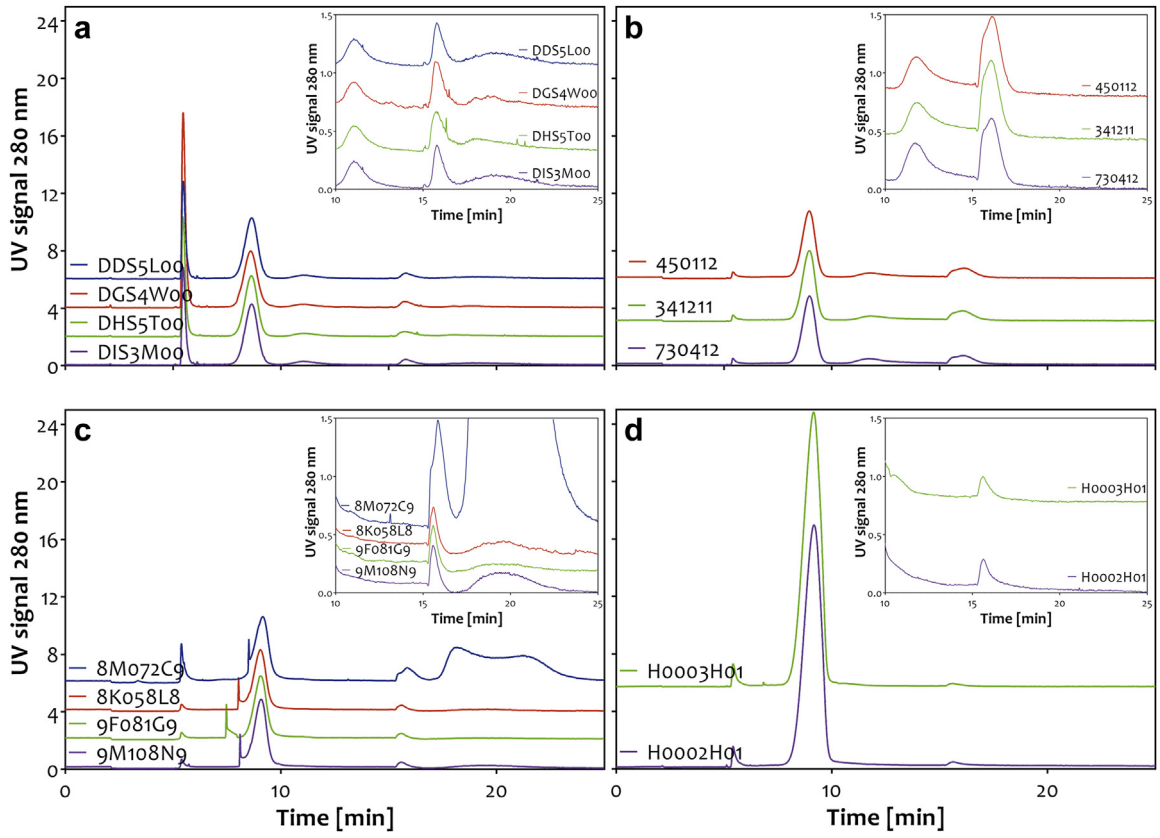

Figure 5. AF4 elugrams of the 4 epoetin products (a) Eprex, (b) Binocrit, (c) Retacrit, and (d) NeoRecormon. The inset is a zoom into the elugrams in the area between 10 and 25 min. The different colors represent the batches of single product.

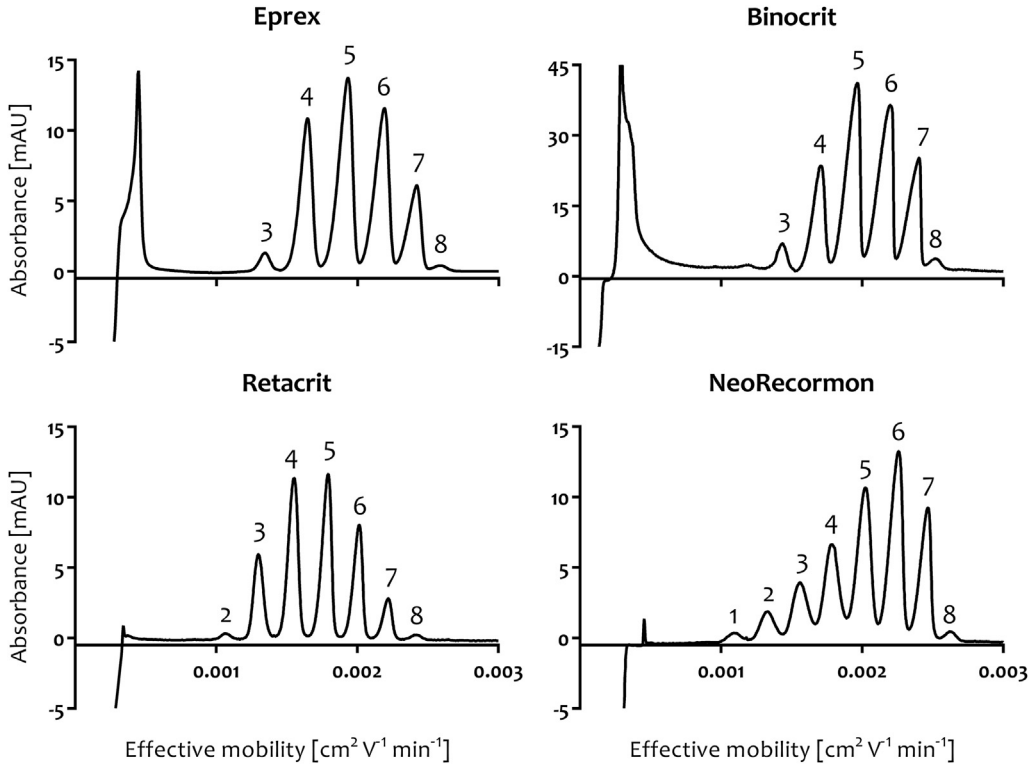

Figure 6. Representative CE-UV analysis of the 4 epoetin products. Each product is represented by 1 repetition of 1 batch.

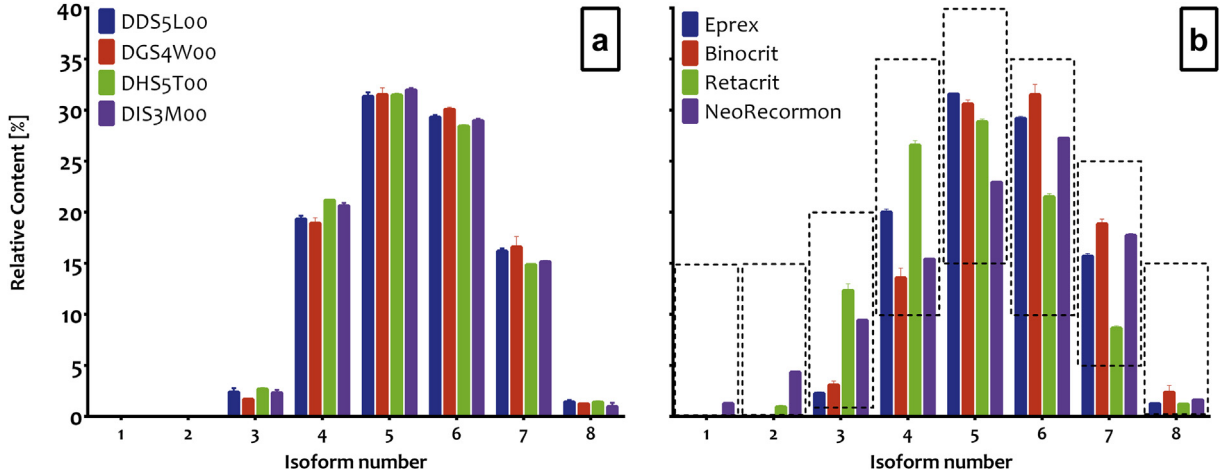

Figure 7. Relative isoform distribution of (a) different batches of Eprex (n = 3) and (b) different epoetin products, namely Eprex (n = 12), Binocrit (n = 8), Retacrit (n = 13), and NeoRecormon (n = 10). The area inside black box represents the acceptance criteria based on Ph. Eur. monograph on Erythropoietin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

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]

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. In addition, the highest content differences between batches ~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 hinders the full mass recovery of the injected protein.[30]

Capillary Zone Electrophoresis

Multiple isoforms of epoetin were detected on separation by CZE 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

Table 4
Weighted Mean Potencies (IU/mL) of 4 Epoetin Products With Upper and Lower 95% Confidence Limits

Product	Batch	Declared Potency (IU/mL)	Epoetin Content as IU/mL (95% Fiducial Limits)		*In Vitro*/*In Vivo*
			In Vitro Potency (IU/mL)	*In Vivo* Potency (IU/mL)	
Eprex	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.
Retacrit	8K058L8	10,000	8610 (7880-9410)	9920 (8234-11,951)	0.87
	8M072C9		9190 (8300-10,200)	N.D.	N.D.
	9F081G9		9440 (9000-9900)	N.D.	N.D.
	9M108N9		9040 (8680-9400)	11,886 (9834-14,365)	0.76
Binocrit	730412	10,000	15,300 (14,300-16,400)	9395 (8190-10,777)	1.63
	4501121		15,200 (14,300-16,400)	8015 (6828-9408)	1.90
	341211		15,700 (14,900-16,600)	8544 (7573-9640)	1.84
NeoRecormon	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.

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).

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 is the highest relative isoform content in Retacrit.[14] Finally, 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]

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]

Discussion

Most previous studies have shown the physicochemical properties of a single batch of multiple epoetin brands only.[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 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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