BIOTECHNOLOGY

Biochemical Assessment of Erythropoietin Products From Asia Versus US Epoetin alfa Manufactured by Amgen

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ABSTRACT: We compared the physical and chemical properties of purported copies of recombinant human erythropoietin (rHuEPO) purchased from Korea, China, and India with the innovator product, Epoetin alfa, manufactured by Amgen Inc. The products were characterized for similarity in the types of glycoforms present, the relative degree of unfolding, *in vitro* potency, presence of covalent aggregates, and presence of cleavage products using established analytical methods. All products were different from Epoetin alfa (Epogen®). The purported copies of rHuEPO from Korea, India, and China contained more glycoforms and other impurities. The *in vitro* relative potency varied for each product when based on the labeled concentration, while the concentration based on ELISA analysis brought the relative potency, for most products closer to 100%. These data emphasize potential biochemical discrepancies resulting from different cell lines and manufacturing processes. Concentrations varied within products and did not always match the information provided on the product label. As it is not possible to reliably correlate such biochemical discrepancies to clinical consequences, or the lack thereof, these data support the need for extensive preclinical testing and clinical testing of all investigational products as not all safety and efficacy aspects can be assessed during preclinical evaluation. J Pharm Sci 98:1688–1699, 2009

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INTRODUCTION

Biopharmaceutical products based on recombinant DNA technology have been on the market since the 1980s. In Europe, the European Medicines Agency (EMEA) has issued specific guidelines allowing for registration and sale of purportedly biochemically similar copies of these products,

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leading several manufacturers to develop them as potential products. These purported copies are known as biosimilars in Europe. The first biosimilar growth hormone was approved in Australia in 2005 and biosimilar rHuEPO was approved in Europe in 2007.

The biochemical and biophysical characteristics of biopharmaceuticals are complex and closely linked to the unique manufacturing processes developed by a manufacturer for each biotherapeutic agent. This has produced a significant challenge to the introduction of biosimilars in Europe and in the United States since the biosimilar manufacturers must develop their own manufacturing processes, including their own cell lines, culture/fermentation conditions, purification procedures, and container closure systems, all of which can affect the product characteristics and stability of the protein within the biopharmaceutical product. Many of these characteristics, such as chemical modifications, secondary and tertiary structure, and aggregation, can potentially affect the clinical characteristics of the product.[1–3] For example, aggregation has been implicated as a potent initiator for inducing an immunogenic reaction and breaking immune tolerance[3–6] and is one of the suspected causes of antibody-mediated pure red cell aplasia (PRCA) associated with Eprex, a rHuEPO.[7]

The difficulty in producing similar biopharmaceutical products even by well established biotechnology companies has been demonstrated multiple times.[8–11] For example, Schellekens and Bausch[12] reported that a manufacturer of interferon beta-1a, produced in Chinese hamster ovary (CHO) cells, showed a significant reduction in immunogenicity when the production site was changed. The batch used for clinical trials produced a 14% incidence of neutralizing antibodies after 12 months of use, while only a 4% incidence was observed with the marketed product. The only difference between the batches was the manufacturing site, and extensive analysis did not show any significant differences in physicochemical characteristics that could influence immunogenicity. In another example related to manufacturing sites for a protein, Jay Siegel, who was previously working at the US FDA's Office of Therapeutics Research and Review in the Center for Biologics Evaluation and Research, reported on a recombinant protein manufactured at sites in Japan and in the West. Pharmacokinetic studies demonstrated significant differences that were subsequently linked, not to ethnic differences, but to small differences in manufacturing lots at 1 of 6 glycosylation sites on the protein.[13]

Most recently, studies by Deechongkit et al.[14] elucidated differences in the tertiary structure, physical stability, and degree of aggregation between rHuEPOs produced by Amgen, manufacturer of Epogen, and Ortho Biotech, manufacturer of Eprex. Importantly, the studies demonstrated that the bulk drug product produced by Amgen and compared to Eprex purchased in Europe contained a differing degree of α-helix content and a different hydrodynamic value (*s* value) as measured by analytical ultracentrifugation. The *s* value is characteristic to each protein and provides information on the overall size and shape of the molecule.[15] The data were especially surprising since the rHuEPOs produced by both companies have identical amino acid sequences and similar glycosylation patterns. A recent publication by Heavner et al.[16] from Johnson and Johnson (Ortho Biotech) confirmed the differences in the s values reported by Deechongkit et al. and further demonstrated the presence of high molecular weight aggregates in the Eprex bulk drug product produced by Ortho Biotech that are absent from the Epogen bulk drug product produced by Amgen.

While the EMEA has recently approved marketing applications for biosimilar rHuEPO, these purported copies of rHuEPO have been available for a number of years in many parts of the world, such as China, Korea, India, and South America. They are sold under a number of different trade names (Tab. 1). The regulatory process required for registration of the products varies from country to country, and in general, the clinical efficacy and safety of these products has not been well characterized. Additionally, Schellekens[8] has demonstrated that multiple heterogeneities exist between analytical characteristics of the different products, leading to the possibility of serious adverse events. Since the initial reporting on the characterization of these purported rHuEPOs by Schellekens,[8] additional manufacturers are producing purported rHuEPOs that are available in multiple countries, such as Russia, Thailand, China, India, Korea, and South America. Because of this and the varied regulatory standards for many countries, we felt it was important to describe characterization of these products that includes additional tests to characterize the integrity of the protein structure.

Table 1. Recombinant Human Erythropoietin (rHuEPO) Sample List from Asia

Marketed Country	Trade Name	Company	Exp. Date	HSA	CHO Cell	Label Conc. (IU)	Lot #	Container Type
USA	Epogen®	Amgen	August 5, 2007	Yes, 0.25%	Yes	2000	P029954	Vial
USA	Epogen®	Amgen	February 2, 2007	Yes, 0.25%	Yes	3000	P008951	Vial
USA	Epogen®	Amgen	January 8, 2007	Yes, 0.25%	Yes	10000	P028155	Vial
Korea	Eporon	Dong-A	February 2007	Yes[a]	Yes	4000	ED50398	Vial
Korea	Eporon	Dong-A	March 2007	Yes[a]	Yes	4000 IU/0.4 mL	PD50908	PFS
Korea	Espogen	LG	November 2007	Yes, 2.5 mg/mL	NA	2000 IU/0.5 mL	EPO05017	PFS
Korea	Epokine	CJ	March 2007	Yes[a]	Yes	4000 IU/0.4 mL	5530	PFS
China	Epiao	SS-Pharm	November 2007	Yes, 0.25%	NA	2000	20051101	Vial
China	Jia Lin Hao	Shandong E- Hua	December 2007	Yes[a]	Yes	3000	20051203	Vial
China	Ji Mai Xin	Hua-Bae Pharm	August 2007	Yes[a]	NA	3000	Y20050931	PFS
China	Ji Mai Xin	Hua-Bae Pharm	September 2007	Yes[a]	NA	3000	Y20051031	PFS
China	Huan Er Bo	Beijing Four Rings	March 2008	Yes[a]	NA	3000 IU/0.6 mL	20060305	PFS
China	Huan Er Bo	Beijing Four Rings	February 2009	Yes[a]	NA	3000 IU/0.6 mL	20060203	PFS
China	SEPO	China-SPG	August 2007	Yes	Yes	4000	20050905	Vial
India	Zyrop	Imported from Argentina (Bio Sidus)	March 2008	Yes, 0.25%	Mammalian cell	10000	H10-4031H01	Lyophilized In vial
India	Wepox	Wockhardt	August 2008	NA	Mammalian cell	40000	XF10336	PFS
India	Shanpoietin	Shantha Biotech	April 2008	NA	Yes	4000	EPO2206	PFS
India	Shanpoietin	Shantha Biotech	July 2008	NA	Yes	4000	EPO2806	PFS
India	Epotin	Imported from China (NCPCGB)	April 2008	Yes	Yes	4000	Y20060541	PFS

NA, not available; PFS, prefilled syringe.
[a]Not listed as an excipient, but listed in precautions.

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MATERIALS AND METHODS

Sample Preparations

Multiple lots of rHuEPO that were readily and commercially available from three Korean companies, five Chinese companies, and four Indian-marketed rHuEPO products were compared against Amgen-manufactured Epogen (Tab. 1). The rHuEPO samples from Korea, China, and India were shipped in temperature-controlled packages (2–8°C) from each country to the United States, where they were stored at 2–8°C for about 1 week before analysis. The samples were analyzed without any manipulation other than that specified for each analytical method. Samples of Epogen were formulated at Amgen Inc. (Thousand Oaks, CA) with 0.25% human serum albumin (HSA), 20 mM sodium citrate, and 100 mM sodium chloride (pH 6.9).

The product inserts from the three products from Korea included the following formulation information: Eporon® and Epokine both contain HSA at unspecified concentrations, and Espogen contains 2.5 mg/mL HSA. The five products from China included the following information on formulation in their respective product inserts: China-Epiao from SS Pharm (Sheng Yang, China) contains 0.25% HSA; Jialinhao from Shandong E-Hua Biotech Pharmaceutical (Shandong, China) contains HSA at unspecified concentrations; and SEPO contains HSA and sodium citrate buffer at unspecified concentrations. Jimaixin, from Hua-Bae (China North) Pharmaceutical, Huan Er Bo from Beijing Four Rings, did not mention HSA in the formulation text, however, it was mentioned in the precautions. For each of the eight products, HSA was either specifically listed as an excipient or listed in the precautions (i.e., patients with a known hypersensitivity to HSA should not receive the product). SEPO was the only product that mentioned the buffer composition for the marketed formulation. The four products from India included the following information on formulation in their respective product inserts: Shanpoietin from Shantha Biotech does not mention HSA; Epotin that is imported from China contains HSA; Zyrop that is imported from Argentina Bio Sidus has 0.25% HSA; and Wepox from Wockhardt does not indicate about HSA.

A number of analytical and biological assays were conducted to compare the Korean, Chinese, and Indian rHuEPO products with Epogen. Details of these assays are described in the following sections.

Isoform Distribution by Isoelectric Focusing With Western Blot and Capillary Zone Electrophoresis

The isoform distribution of samples was analyzed by isoelectric focusing (IEF) analysis using standard laboratory methods. The samples were subjected to polyacrylamide gel IEF in a pH 3–5 gradient. Urea (6 M) served as denaturant. Gels were prefocused at 2000 V for 20 min, samples were applied, and electrophoresis was performed at 2000 V for approximately 2.5 h. Gels were subjected to Western blotting on polyvinylidene fluoride (PVDF) membranes, and immunohistochemical visualization of proteins was conducted as described below.

The reference standards from specific lots of Amgen's Epogen bulk and samples of rHuEPO were separated by capillary zone electrophoresis (CZE). CZE analysis was performed using aneCAP amine-coated capillary (50 μm internal diameter × 50 cm effective length) and a Beckman MDQ. Samples were concentrated to 1.0 mg/mL using a 30K microconcentrator and MilliQ water. The injection was performed at 0.5 psi for 15 s. Separation was accomplished using 8 M urea in 300 mM phosphate buffer at −12 kV for 45 min. Detection was at 200 nm.

Aggregation or Degradation Detection by SDS–PAGE With Western Blot

Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Western blot according to standard techniques to detect aggregation or degradation of samples. Samples were separated on 10–20% gradient Tris-Glycine gels (Novex, Carlsbad, CA), and blotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). Protein products were visualized by Western blot analysis. Blots were blocked for 16 h in 10% horse serum (HS) in phosphate-buffered saline (PBS, pH 7.4, Gibco, Grand Island, NY). The blots were incubated with an Amgen-produced monoclonal mouse anti-Epoetin antibody (diluted 1:100) for 3 h, washed with PBS, and then incubated with a biotinylated anti-mouse secondary antibody (diluted 1:650, Vector Laboratories, Burlingame, CA). All antibody dilutions were prepared in PBS containing 5% HS. The blot was incubated with an avidin:-horseradish peroxidase (HRP) conjugate (diluted

1:2300, Reagents A and B from Vectastain ABC kit, Vector Laboratories), and final visualization of proteins was through chromogenic detection by reaction of HRP with HRP color development reagent (Bio-Rad, Hercules, CA). Images were captured on a Bio-Rad GS-800 imaging system using Quantity One® software.

rHuEPO Structure Conformation by 9G8A Assay

Conformational similarity of the product samples was assessed using a 9G8A antibody binding assay.[17,18] The 9G8A monoclonal antibody recognizes a linear epitope, ERYLL, consisting of amino acids 13–17, in both native and denatured EPO. These amino acids become more exposed following a conformational change in structure or denaturation of EPO, allowing for increased 9G8A binding. Samples were diluted to the range from 0.34 to 4.2 μg/mL, depending on the sample, in 1× PBS, 1% BSA, 0.1% polysorbate-80 buffer, and distributed into a 96-well plate. Fifty microliters of TAG (BioVeris, Gaithersburg, MD) labeled 9G8A (2 μg/mL, prepared by Amgen, Process & Analytical Sciences) anti-rHuEPO antibody was added, and the samples were incubated for 1 hour at room temperature on a plate shaker. Then, 50 μL of an affinity-purified, biotinylated rabbit anti-rHuEPO antibody (stock concentration 0.5 μg/mL) was added, and the samples were incubated for 1 h at room temperature on the plate shaker. Next, 25 μL of Streptavidin-coated beads (stock concentration, 0.566 mg/mL) diluted to 0.6 μg/mL in 1× PBS, 1% BSA, and 0.1% polysorbate-80 were added, and the samples were incubated for 30 min at room temperature on the shaker plate. The plate was then transferred to an M8 analyzer (IGEN International, Gaithersburg, MD), and the chemiluminescent signal was measured according to the manufacturer's instructions. The chemiluminescent signal was converted to a ratio of sample to Epogen bulk standard and reported as relative reactivity, with 1 being equal to the standard and values >1 indicating increased protein denaturation.

rHuEPO Concentration by ELISA and Potency By *In Vitro* Bioassay

A solid-phase sandwich ELISA was performed with the Quantikine IVD Human EPO Immunoassay Kit (#DEP00, R&D Systems, Minneapolis, MN) to quantify the concentration of rHuEPO in test samples. Microtiter plates precoated with murine monoclonal antibody specific for rHuEPO were initially incubated with 1% BSA in 1× PBS Blocking Buffer. Following incubation and a wash step, serially diluted test samples, standards and controls, and Assay Diluent were added to the microtiter plates. An Amgen rHuEPO Final Dosage Form (FDF, Drug Product) Reference Standard and an Amgen-released lot of rHuEPO served as the standard and control, respectively, for the assays.

rHuEPO in the test samples, standards, and controls bound to the monoclonal antibody specific for rHuEPO that was already immobilized on the microtiter wells. After incubation, excess test samples, standards, and controls were removed with a wash step, and a Rabbit anti-EPO polyclonal antibody conjugated to horseradish peroxidase solution was added to the microtiter plates. Following incubation, excess conjugate was removed with a final wash step and a chromogen was added. Oxidation occurred between the chromogen and horseradish peroxidase enzyme, allowing the optical density of the contents of each of the microtiter wells to be measured. The amount of color measured as optical density is directly proportional to the amount of rHuEPO in the test samples. Plates were read on a Powerwave HT (Biotek, Winooski, VT) at 450 nm primary wavelength, 650 nm reference wavelength, and sample concentrations were calculated using a dose–response curve.

A proprietary gene expression bioassay method developed at Amgen utilizing an erythropoietin-dependent human leukemic megakaryocyte cell line was used to determine the *in vitro* potency of product samples. Samples were tested in a relative potency format using the product concentrations stated on the labels and compared to an Amgen Epogen reference standard. Briefly, cells plus samples were incubated at 37°C for approximately 4 h and then treated with a lysing detergent and luciferin as a substrate. The luminescence resulting from the reaction of luciferase with luciferin was measured using a luminometer model 1450 Microbeta Trilux (Perkin Elmer, formerly Wallac, Waltham, MA). Test sample activity was determined by comparing the test sample response to the response obtained with the Amgen Epogen Reference Standard.

pH Measurement

pH was measured using a Mettler Toledo MP220 pH meter with 100 μL of sample.

Osmolarity Analysis

Osmolarity of each sample was measured using an osmometer from Advanced Instruments Inc. (Model 2020, Norwood, MA).

RESULTS

Isoform Analysis

IEF-Western blot and the complimentary CZE analysis for rHuEPO separates charge isoforms based on the number of sialic acid residues on each protein. Each rHuEPO may have up to three specific Asn residues (Asn 24, 38, 83) glycosylated and 1 Ser residue (Ser 126) glycosylated. The glycans on the rHuEPO are branched chain glycans with up to 4 sialic acid residues capping each glycan on the Asn residues and up to 2 sialic acid residues capping the glycan on the Ser, for a total of 14 sialic acids in a fully glycosylated protein. A fully glycosylated protein is known as isoform 14. During production of the rHuEPO in the CHO cells, each protein is glycosylated to a differing degree, ranging from partially to fully glycosylated. The lower-charged isoforms are removed during the manufacturing and purification process; however, the degree of removal is dependent on the purification process employed being different for each manufacturer.

The isoform pattern for Epogen was consistently observed across product batches. Analysis of EPO isoforms in rHuEPO product purchased from China and Korea showed a high degree of isoform variability in the samples (Fig. 1). Product from Jia Lin Hao (lane 4), Ji Mai Xin (lanes 5 and 6), and Huan Er Bo (lanes 7 and 8) purchased from China showed approximately 9 isoforms. Additionally, the isoform patterns differed between each of the two batches of Huan Er Bo, indicating differences in the manufacturing process of the product within the same company. The isoforms for Eporon (lanes 10 and 12) from Korea were more than for Epogen and also inconsistent between product batches. The rHuEPO samples from India showed multiple isoforms and unknown species as compared to the Epogen sample from Amgen (Fig. 1B). In particular, Zyrop that is manufactured in Argentina and sold in India and Wepox, which is manufactured in India, showed the presence of multiple lower-charge isoform species, indicating either degradation during storage or incomplete removal of the lower-charge species of the protein during the manufacturing process. These results were repeated and confirmed (data not shown).

CZE analysis was used to further characterize the isoform distribution of rHuEPO samples (Fig. 2A–C). The CZE profile of Epogen was consistent with findings from the IEF analysis, with four distinct isoforms detected (corresponding to isoforms 10, 11, 12, and 13) without any notable impurities apparent in the region preceding elution of the isoforms. As illustrated in Figure 2A and B, differences exist in the isoform distributions of all the samples of Korean, Chinese, and Indian rHuEPOs compared with the control (Epogen). The Ji Mai Xin purchased from China and Wepox, Zyrop, and Epotin purchased from India showed additional later-eluting isoform peaks compared with Epogen and deviations of the electropherogram to baseline from 30 to 34 min on the electropherogram. These results correspond with the IEF data. The CZE profiles of Eporon, Espogen, Jia Lin Hao, Huan Er Bo, and SEPO also differed from Epogen with respect to the number of isoforms and to

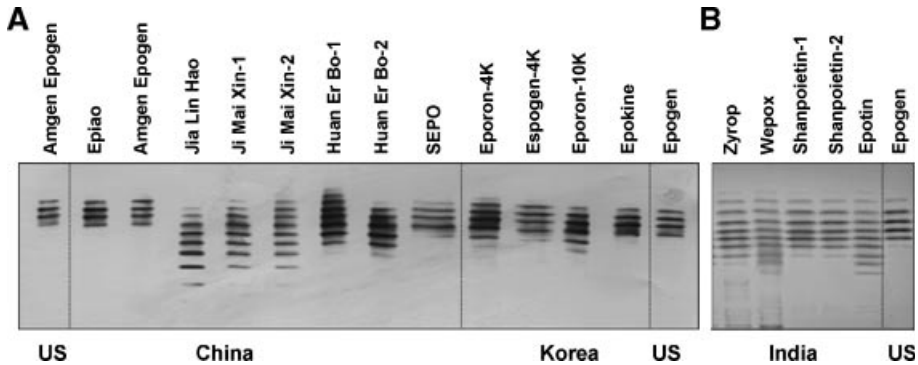

Figure 1. Iso-electro-focus (IEF) Gel with Western blots for isoform detection: (A) samples from China (lanes 2–9) and Korea (lanes 10–13) and (B) samples from India (lanes 1–5).

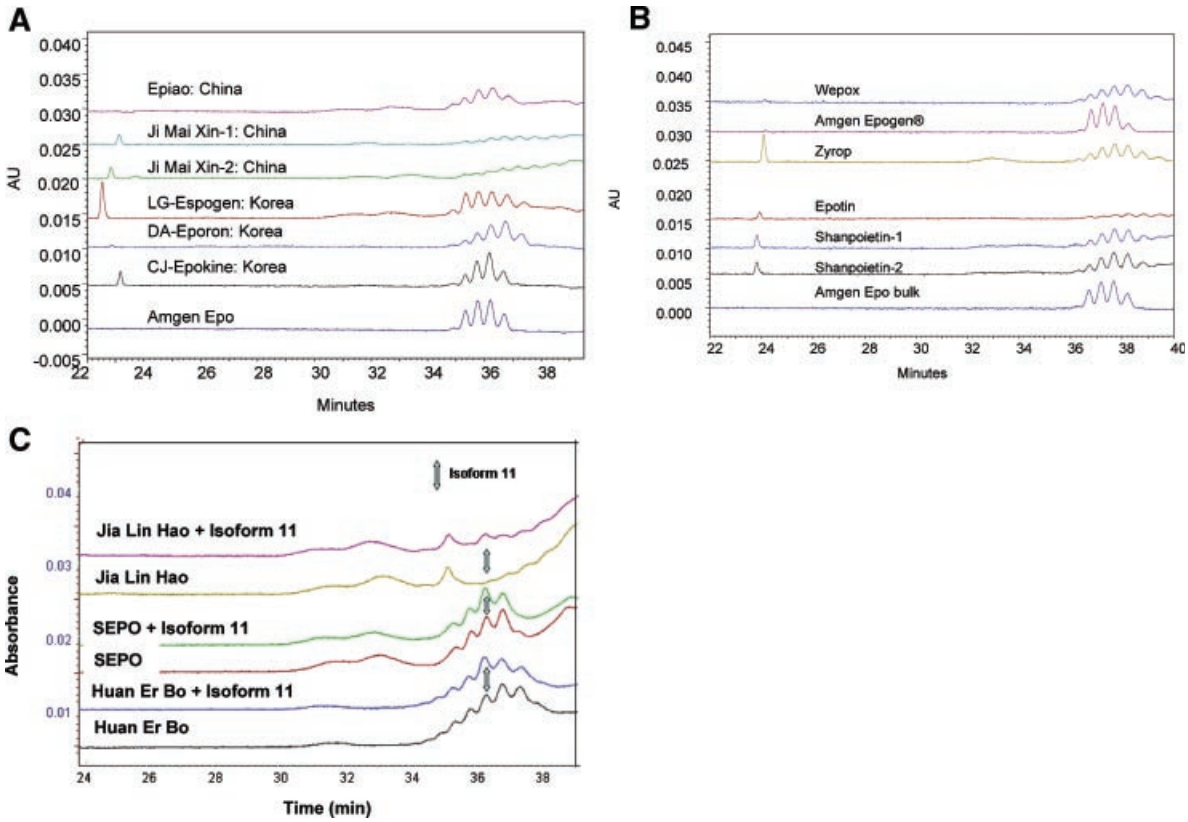

Figure 2. Capillary zone electrophoresis of rHuEPO: (A) from China and Korea, (B) India, and (C) Chinese EPO samples with isoform 11 spiked to confirm isoform 11 position.

impurities for some products. In particular, the isoform pattern for the sample from Jia Lin Hao was dramatically different compared with the Epogen bulk from Amgen. The identity of the isoforms was confirmed by spiking isoform 11 into each sample and comparing them to the unspiked samples (Fig. 2C).

rHuEPO Sample Degradation and Other Impurities

Nonreducing SDS–PAGE Western Blot analysis demonstrated a single band at approximately 36–40 kDa for Epogen (Fig. 3). The Eporon samples from Korea, (lanes 4 and 5), showed a smear of high molecular weight bands indicative of large protein aggregates while the Epokine sample from Korea, Epokine (lane 7), showed the presence of a band with a molecular weight expected for a dimer. A faint signal for these bands was also present in the Ji Mai Xin and SEPO samples from China and the Shanpoietin samples from India. Wepox from India (lane 19) showed several additional bands above the main band, indicative of covalently cross-linked aggregated species. Zyrop (lane 18) purchased from India but manufactured in Argentina showed several additional bands above the main band, also indicative of protein aggregation.

The 9G8A monoclonal antibody recognizes a linear epitope consisting of amino acids 13–17.[18] Based on the NMR structure of the *E. coli* produced MKLysEPO, the side chains of residues 15 and 16 are internal to the protein[19] and unavailable for detection by the antibody. Following a conformational change or unfolding of the rHuEPO the residues become exposed to the solvent, allowing for detection of the epitope by the 9G8A antibody. The data is plotted as a ratio of reactivity of the sample to the reactivity of an Epogen bulk standard. Fully folded rHuEPO has a value of 1. Unfolding is defined as an increase in the value. The majority of the biosimilars from China and Korea showed reactivities greater than 1 (Fig. 4), indicating denaturation of some portion of the protein. The values were highest for Wepox, with a value of 14. Eporon-4K was highest among Korean samples, and Huan Er Bo-1 sample was highest

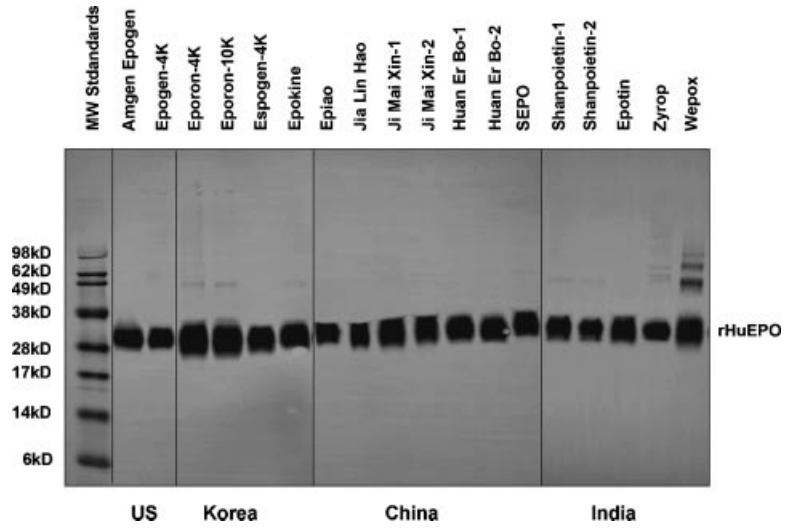

Figure 3. SDS–PAGE with Western blot analysis for detection of aggregation.

among Chinese samples. Lot-to-lot variability was also evident in the degree of 9G8A reactivity between samples of Huan Er Bo. A change in the manufacturing process, handling, and storage could introduce contaminants and may result in changes in protein folding.

rHuEPO Concentration and *In Vitro* Potency

Concentration analysis by ELISA confirmed the label concentration for a number of the rHuEPO products, but it also indicated a difference between the concentration indicated on the label (bar with stripe) and that measured by ELISA (bar with color) for Dong-A Eporon from Korea and Wepox and Zyrop from India. The majority of the Asian rHuEPO samples had a higher concentration than what was stated on the label (Fig. 5), with the Eporon-4K sample showing a value approximately 80% higher than that stated on the label and Eporon-10K and Wepox values being 70% higher (Tab. 2). The measured relative potency values obtained using the *in vitro* potency assay also demonstrated that most exceeded the label concentration. When these values were adjusted based on the ELISA concentration data, most were at 100%. Wepox had the lowest ELISA adjusted relative potency value of 78%, whereas the 2 Shanpoietin samples had the highest values.

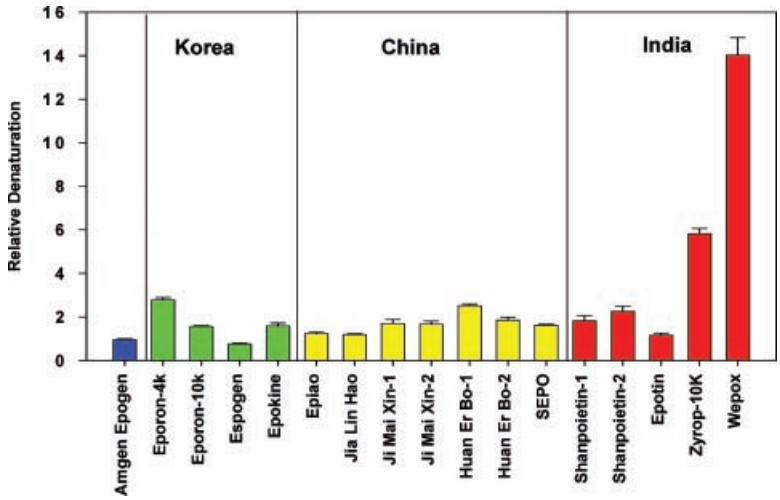

Figure 4. Relative denaturation by 9G8A antibody assay to detect unfolding structure: Samples from China, Korea and India were compared to Amgen Epogen. A value of 1 indicates no difference in folding between the sample and the EPO standard.

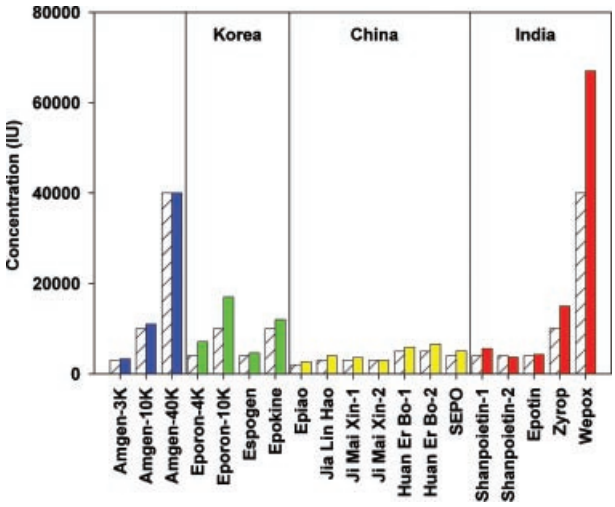

Figure 5. Concentration determination by enzyme-linked immunosorbent assay (ELISA). Striped bars represent the labeled concentration and solid bars represent the concentration measured by ELISA.

Osmolarity and pH Analysis

A pH of 6.9 was indicated on the majority of sample labels for the products. When we considered the specification of 6.9 ± 0.3, pH 6.6 to pH 7.2 was acceptable for these samples. Most of samples were in this range of 6.9 ± 0.3. However, one lot of Shanpoietin from India was higher than pH 7.2, and the other lot of Shanpoietin from the same company showed a pH of 6.9 (Tab. 2). Osmolarity analysis also demonstrated problems with the quality of the products. An isotonic solution is approximately 300 mOsm/kg. One lot of Shanpoietin from India with a pH of 7.3 showed a value of 27 mOsm/kg, hypotonic by osmolarity analysis, while the other lot showed 220 mOsm/kg. The result was checked 3 times and compared to a standard to confirm its accuracy (Tab. 2). The majority of products showed osmolarities between 200 and 300 mOsm/kg, being isotonic to biological fluids. Espogen purchased from Korea and Wepox purchased from India showed values higher than 300 mOsm/kg, indicating hypertonicity of the products.

DISCUSSION

In agreement with previous findings,[8] the results of this investigation confirm that epoetin products from different manufacturers can differ widely in biochemical composition. These study results do not necessarily imply that the rHuEPO products from Asia studied here are clinically inferior to the innovator agent. However, these data underscore

Table 2. Measured Values of pH, Osmolarity, *InVitro* Potency, and Concentration of rHuEPO Samples from Asian Market

Marketed Country	rHuEPO Samples	pH	Osmolarity (mOsm/kg)	Potency[a] by Bioassay (IU/mL)	Concentration Based on Label (IU/mL)	Concentration by ELISA (IU/mL)	Measured[b] Concentration as % of Label Value
Korea	Eporon-4K	6.91	274	6200	4000	7100	180
	Espogen-4K	6.97	348	4100	4000	4600	120
	Eporon-10K	6.94	274	14000	10000	17000	170
	Epokine-10K	6.89	239	10000	10000	12000	120
China	Epiao-2K	6.80	241	2600	2000	2700	140
	Jia Lin Hao-3K	6.80	279	4100	3000	4000	130
	Ji Mai Xin3K-1	6.79	245	3300	3000	3600	120
	Ji Mai Xin3K-2	6.83	245	3000	3000	3000	100
	Huan Er Bo5K-1	6.75	291	5300	5000	5900	120
	Huan Er Bo5K-2	6.70	244	6200	5000	6600	130
	SEPO-4K	6.87	251	4400	4000	5100	130
India	Zyrop10K	7.19	291	15000	10000	15000	150
	Wepox40K	6.73	334	52000	40000	67000	170
	Shanpoietin4K-1	6.91	220	7000	4000	5600	140
	Shanpoietin4K-2	7.25	27	4500	4000	3700	93
	Epotin4K	6.88	240	4700	4000	4300	110
USA	Epogen®	6.88	246	3000	3000	2900	97

[a]Relative potency determined as compared to Amgen Reference Standard.
[b]100 × concentration by ELISA/concentration based on label.

the variability that exists among biopharmaceuticals and form the basis for a strong argument in favor of consistently high standards of quality control in product manufacturing and processing. The data also underscore the fact that a single assay cannot adequately demonstrate comparability between drug products such that multiple, well-designed validation assays are essential to ensure product purity and lot-to-lot consistency.

The basis for the discrepancies among the product samples analyzed during this study is unknown, but it is possible that these differences could be attributed to the differing manufacturing processes.[20,21] Indeed, Deechongkit et al.[14] demonstrated that the active proteins in Epogen and Eprex, Epoetin alfa produced by the manufacturers Amgen and Ortho Biologics LLC, were not identical when compared using well-known biophysical analytical techniques. Manufacturing processes for recombinant proteins, such as production cell lines, culture conditions, purification methods, formulation type and ingredients, contaminant profiles, and packaging components all contribute to the characteristics of the end product. With many of these methods being proprietary by nature, it is not surprising that the end products differ among various manufacturers. The specific formulation information for most of the Asian rHuEPO products is imprecise at best in the product inserts. This was exemplified by the product inserts for the Asian rHuEPO's we examined in which the product descriptions ranged from no comment on excipients to a full description of all excipients. Based on our own analysis by SDS–PAGE and reverse phase HPLC analysis (data not shown) the products all contain albumin. Further confounding the issue is the possibility that different manufacturers may utilize different bioassays and/or different standards to determine the specific activity of their rHuEPO products. This problem is illustrated by the potency values shown in our studies in which large differences were observed when the potency was determined based on the labeled concentration for each product.

The data reported here confirm that there is potential for the introduction of biochemical differences among recombinant protein therapeutics produced by different manufacturers. Large differences between reported concentrations and actual manufactured concentrations may lead to inadvertent dosing miscalculations by doctors without their knowledge. The concentration ambiguities, the presence of impurities, and the overall product heterogeneity issues observed in this study have unknown clinical consequences. Small differences between recombinant protein products have been shown to be clinically relevant, particularly with regard to immunogenic potential.[22–24] For example, a small change in the formulation of a rHuEPO product, during which HSA was replaced with polysorbate 80, corresponded with a notable increase in the incidence of antibody-mediated PRCA.[22] The etiology of this immune response is unclear, as a number of factors are known to contribute to the immunogenicity of recombinant proteins.[1,25]

The presence of high molecular weight aggregates of the proteins has been linked to immunogenicity in a number of studies.[3–6] The general rules governing the requirements for aggregates producing an immunogenic response were elucidated by Dintzis et al.[26] and showed that an aggregate of at least 12 monomers may be necessary to cause an immune response, albeit the propensity to cause a response will be highly protein and aggregate dependent. Espogen, Eporon and Epokine (Fig. 3) showed the presence of high molecular weight species unable to enter the polyacrylamide gel used for separation of the proteins. While the size of the aggregates was not determined, proteins of at least 200 kDa were separated and indicated that the covalently cross-linked aggregates were of much greater molecular weight. It is also possible that noncovalently cross-linked aggregates were also present but could not be demonstrated by this technique. In addition to aggregation, protein unfolding has also been linked to immunogenicity,[1] as this would lead to exposure of epitopes previously buried within the interior of the protein. In our studies some degree of unfolding was demonstrated by the reactivity of the 9G8A antibody against many of the purported recombinant erythropoietins (Fig. 5). By this assay the buried residues 13–17 must be exposed to the solvent to be recognized by the antibody. The full significance of the unfolding could not be determined as we are currently unable to estimate the percentage of unfolded protein detected in the assay. Potency of epoetins is determined in part by the isoform distribution present in the product. Measured differences in the *in vitro* potency values for these products based on label concentrations are complicated by the fact that the isoform distribution is not identical to Epogen and that some contain aggregates and degradants as well.

Although it is not known if manufacturers of the Asian rHuEPO products evaluated in this study are currently seeking entry into the EU or US market, the data presented here highlight the difficulties facing both biosimilar manufacturers and regulatory authorities. Biopharmaceuticals are, by their very nature, more complex to characterize than small-molecule drugs. This is reflected in the recent guidelines issued by the EMEA for the approval of biosimilars.[27–31] The EMEA recognizes that biosimilars will be similar but not identical to reference products. As the clinical consequences of these biochemical differences cannot be reliably predicted based on laboratory analyses alone, the EMEA requires randomized controlled trials to demonstrate the biosimilar has similar efficacy and safety to that of the innovator. This was exemplified by the recent marketing authorization of three biosimilar Epoetin alfa products (Abseamed from Medice Arzneimittel Pütter, Binocrit from Sandoz Pharmaceuticals, and Epoetin alfa Hexal from Hexal Biotiechnology) by the EMEA (EPAR 2008). All three biosimilars are produced by Rentschler Biotechnology GmbH in Germany. Because of the risk of immunogenicity and rare adverse events, biosimilars will also require rigorous postmarketing surveillance. For example, Eprex, which has recently been re-approved for subcutaneous injection in Europe and Australia, is carrying out a postmarketing surveillance study to estimate the incidence of antibody-mediated PRCA associated with the product as compared to the other ESA's currently on the market.[32]

In conclusion, biophysical differences between recombinant proteins cannot predict clinical outcomes. These can only be assessed through comparative clinical trials that are designed specifically to detect clinically meaningful differences between the biosimilar and the innovator. The EMEA has shown that such clinical studies can indeed be designed, conducted, and shown to be capable of detecting, and therefore enabling rejection of, products that fail to meet the appropriate standards of similarity. Such has been the case with a biosimilar interferon alfa-2a that was rejected by the EMEA on the multiple grounds of having failed to demonstrate similarity, but most notably that more patients experienced a relapse of their disease than patients who received the innovator. Such a conclusion could only be drawn following the conduct of a comparative clinical study and could not have been predicted or based solely on biophysical or preclinical data.

As more biosimilar products come to market, it is essential that clinicians, pharmacists, and patients become more aware of the similarities and differences between biosimilars and reference products, as well as the potential clinical consequences of any such differences. Regulatory authorities have a responsibility to establish an appropriate paradigm for the proper preapproval clinical evaluation and use of biosimilars to ensure that patients are not exposed to unnecessary risks.

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