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Development of a sensitive size exclusion HPLC method with fluorescence detection for the quantitation of recombinant human erythropoietin (r-HuEPO) aggregates

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Abstract

Human erythropoietin produced by recombinant DNA technology, is now marketed worldwide for the treatment of anemias associated with chronic renal failure and chemotherapy. No sensitive methods, which can determine r-HuEPO dimer or oligomer aggregate content in formulated products, have been published to date. This report describes the development and validation of a sensitive size exclusion high performance liquid chromatography (HPLC) method for the quantitation of r-HuEPO aggregates in formulations containing 0.03% polysorbate 80. A Waters Alliance 2690 HPLC system connected to a TosoHaas TSKgel G3000 SWxl (7.8 mm × 30 cm, 250 Å pore size, 5 μm particle size) column and a Waters 474 fluorescence detector was used. The mobile phase for the SEC-HPLC method consists of isopropyl alcohol–potassium phosphate (0.1 M)/potassium chloride buffer (pH 6.8 ± 0.1, 0.2 M) (25:75, v/v). The flow rate was 0.3 mL/min and the method run time was 60 min.

The SEC-HPLC method presented here was shown to be specific for r-HuEPO total aggregates (dimer and oligomers) and allows for their quantitation at 80 ng/mL or 4 ngs/injection, in the presence of r-HuEPO monomer and the pharmaceutical excipients, glycine (5 mg/mL), sodium chloride (4.3 mg/mL), and 0.03% polysorbate 80. The finalized method is stability-indicating and is suitable for determining r-HuEPO aggregates between 0.2 and 0.5% levels in the formulated product of r-HuEPO. This method offers a robust way to measure total aggregates on a routine basis with a high sensitivity for use in product quality control.

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1. Introduction

Erythropoietin (EPO) is a glycoprotein that is produced primarily by the kidneys and is a main hormone involved in regulating red blood cell production [1]. The protein has a molecular mass of 34 kilo Daltons (kDa) with carbohydrates comprising 40% of the weight. In 1986, EPO was successfully expressed by applying recombinant DNA technology [1–3]. Recombinant human erythropoietin (r-HuEPO) is now marketed worldwide for the treatment of anemias associated with chronic renal failure and chemotherapy [4].

The EPO molecule has been well-characterized [5–9] and is a stable molecule that remains predominantly in monomeric form when stored at 2–8 °C. However, when the product is exposed to higher temperatures or to certain stress conditions, dimer and higher order aggregates of r-HuEPO can be formed [10,11]. As with many other marketed biopharmaceuticals, to protect the active protein against denaturation or aggregate formation, non-ionic surfactants such as polysorbate 80 are included as stabilizers [12–15]. The stability of the EPO protein can be monitored by size exclusion high-performance liquid chromatography (SEC-HPLC), which resolves the aggregate forms of r-HuEPO from r-HuEPO monomeric protein. However, polysorbate 80 present in some formulations can interfere with aggregate quantitation because its peaks elute at similar retention times to that of the r-HuEPO aggregates. This interference becomes significant under sensitive detection conditions and complicates the development

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of sensitive and quantitative SEC-HPLC methods capable of quantitating r-HuEPO aggregates in formulated product at very low levels (∼0.2%).

Although analytical HPLC methodologies have been described previously, they have been developed either for analysis of purified r-HuEPO monomeric protein [16–18] or for investigation into r-HuEPO (monomer) metabolic pathways [19]. No sensitive methods for measuring r-HuEPO dimer or oligomer aggregates in formulated products have been published to date. This report describes the development and validation of a sensitive SEC-HPLC method for the analysis of r-HuEPO aggregates.

The SEC-HPLC method presented here was shown to be specific for r-HuEPO aggregates in the presence of r-HuEPO monomer and the formulation excipients glycine (5 mg/mL), sodium chloride (4.3 mg/mL), and 0.03% polysorbate 80. The validated SEC-HPLC method described here allows for the quantitation of r-HuEPO aggregates at 80 ng/mL or 4 ngs/injection in the presence of 0.03% polysorbate 80.

2. Methods

2.1. Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, polysorbate 80, sodium chloride, glycine, guanidine and urea were purchased from Sigma (St. Louis, MO, USA). HPLC-grade water, methyl alcohol and isopropyl alcohol were purchased from Burdick and Jackson (Muskegon, MI, USA). Trifluoroacetic acid (TFA) and triethyl amine (TEA) were purchased from Pierce Chemicals. Recombinant human erythropoietin produced at a commercial manufacturing site was used. Purified r-HuEPO, at 3.1 mg/mL, was transferred into vials and stored frozen at −70 °C until used.

2.2. Chromatography conditions

The analyses of r-HuEPO aggregates by SEC-HPLC (referred to as SEC-HPLC method) were performed using a Waters Alliance 2690 HPLC system connected to a TosoHaas TSKgel G3000 SWxl (7.8 mm × 30 cm, 250 Å pore size, 5 μm particle size) column and a Waters 474 fluorescence detector. The chromatographic control and data acquisition and analysis were performed using Empower software via a Waters LACE data acquisition box. The samples were stored refrigerated in an auto sampler, which is part of the Waters Alliance 2690 Alliance system. The fluorescence detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 345 nm, at a gain of 1000. The mobile phase for the SEC-HPLC method consists of isopropyl alcohol-potassium phosphate (0.1 M)/potassium chloride buffer (pH 6.8 ± 0.1, 0.2 M) (25:75, v/v). The mobile phase was filtered using a 0.45 μm filter (Millipore) and was degassed with an online degasser. The flow rate was 0.3 mL/min and the column was maintained at ambient temperature. The method run time was 60 min. The injection volumes for test samples and standards varied between 50 and 100 μL depending on the sample concentration. The column was equilibrated with a minimum of 5 injections (50 μL) of sample diluent buffer (see Section 2.4) until a stable baseline was obtained.

An existing, second SEC-HPLC method (referred as SEC-HPLC-2 method), for comparison, used a TosoHaas, 30 cm × 7.5 mm, G3000 SWxl column. The SEC-HPLC-2 method used citrate buffer (pH 7.0, 20 mM) with 0.1 M NaCl at as the mobile phase and did not include isopropyl alcohol. The mobile phase was filtered using a 0.45 μm filter (Millipore) and degassed with an online degasser. The samples were injected at 100 μL injection volume, at a concentration of 1.0 mg/mL, and the mobile phase flow rate was 1.0 mL/min. The chromatographic detection was performed using Waters PDA detector (UV at 280 nm). The SEC-HPLC-2 method was qualified for the analysis of samples that did not contain polysorbate 80.

2.3. Reagents preparation

2.3.1. Preparation of sample diluent buffer (SDB)

The sample diluent buffer is prepared in two steps: (A) prepare potassium phosphate (0.1 M)/potassium chloride buffer (pH 6.8 ± 0.1, 0.2 M) and (B) dilute formulation placebo 3-fold with the phosphate buffer that was prepared in step A. The SDB final solution contained 0.01% polysorbate 80 to minimize non-specific adsorption to the vials. The formulation placebo solution had the following composition: sodium chloride (4.3 mg), sodium dihydrogen phosphate (1.16 mg), disodium phosphate dihydrate (2.23 mg), glycine (5.0 mg) and polysorbate 80 (0.30 mg) in 1 mL of water for injection with a pH 6.8 ± 0.1.

2.3.2. Preparation of heat induced r-HuEPO aggregates

Heat induced r-HuEPO aggregates were prepared for use as a standard, for r-HuEPO quantitation, by heating the r-HuEPO solution in 50 mM citrate buffer at 1 mg/mL concentration for about 2 weeks at 50 °C on a heating block. The resulting aggregates were then characterized by SEC-HPLC-2 method, and the oligomer, dimer, and monomer r-HuEPO content was determined for each lot. From this r-HuEPO heat induced aggregate stock solution, working aggregate controls were prepared with concentrations in the range of 0.05–4.0 μg/mL in SDB for the purpose of generating an aggregate control curve. Aggregate controls prepared this way are stable for 3 days (76 h, at 5 °C) and were analyzed within that time frame.

2.3.3. Preparation of r-HuEPO samples spiked with r-HuEPO heat induced aggregates

Samples of r-HuEPO that contained r-HuEPO at 16.67, 33.33, 83.33 and 333.33 μg/mL strengths in formulation placebo were spiked with heat induced r-HuEPO aggregates at LOQ (0.2–0.5%) levels. Spiked samples were prepared by first adding the formulation placebo to an empty polypropylene vial, followed by the r-HuEPO stock solution, and then by heat induced r-HuEPO aggregate stock solution. These solutions were then mixed by gentle inversion (15×) to ensure homogeneity.

2.4. Optimization of isopropyl alcohol content in the mobile phase

The following co-solvents and organic modifiers were tested (at levels up to the value indicated in parentheses) to identify additives that would minimize placebo interference: methanol (10% v/v), isopropyl alcohol (25% v/v), trifluoroacetic acid (0.5% v/v), triethylamine (0.5% v/v); guanidine HCl (2 M), urea (2 M), and polysorbate 80 (0.03% w/v).

2.5. r-HuEPO aggregate stability in the presence of 25% isopropyl alcohol

The stability of the heat induced r-HuEPO aggregates in the presence of the 25% isopropyl alcohol containing mobile phase was investigated by comparing the percent oligomer, dimer, and monomer compositions of 5 r-HuEPO aggregate lots by two different SEC-HPLC methods (see Section 2.2).

2.6. Linearity, recovery and precision

2.6.1. Linearity

The peak area linearity of the heat induced r-HuEPO aggregates with concentration was determined by independently preparing 6 aggregate controls between 0.05 and 4.0 μg/mL, five times each and testing them. The total aggregate area (dimer and oligomer peak areas) was plotted against the concentration (μg/mL) to generate the r-HuEPO total aggregate control curves. Linear regression analysis of the peak area was performed on these curves and the curve's slopes, intercepts, correlation coefficients, and percent relative standard deviations (% R.S.D.s) were determined using Microsoft Excel software. As a system suitability criterion, the correlation coefficient (r^2) had to be greater than or equal to 0.995. The control curve slope (m), y-intercept (c) and the unknown sample fluorescence values (x) were used to calculate the concentration of the aggregates (x), using the equation $y = mx + c$. The limit of detection (LOD) was established as a concentration, at a signal to noise ratio of 1.5 or greater, at which one can reliably detect dimer and oligomer aggregate peaks. The limit of quantitation (LOQ) was established as a concentration, at a signal to noise ratio of 2.5 or greater, at which one can reliably quantify dimer and oligomer aggregate peaks.

2.6.2. Injection precision

Injection precision was determined at the limit of detection (LOD; 0.05 μg/mL) and at the limit of quantitation (LOQ; 0.08 μg/mL) for total aggregates (dimer and oligomer) by injecting six times, from the same vial, and calculating the percent R.S.D.

2.6.3. Recovery and precision

Heat induced r-HuEPO aggregate material was spiked into formulation placebo that contained r-HuEPO (see Section 2.3.3) and the recoveries were determined as a measure of accuracy. The total aggregate peak areas of aggregate spiked test samples, the slope and intercept of the heat induced aggregate control curve (0.05–4.0 μg/mL) were then used to calculate the recovered concentration of the aggregates in spiked samples. The percent recovery values were then calculated for each spiked sample tested by expressing the aggregate concentration as a percent of the r-HuEPO strength of the spiked sample. Inter- and intra-assay precision on r-HuEPO samples spiked with aggregates at four concentrations were determined by testing in triplicate on 3 different days on different machines, operators and the % R.S.D. were determined.

2.7. Stability-indicating capability

The stability-indicating capability of the method was determined by testing r-HuEPO samples, at concentrations ranging from 16.67 to 333.33 μg/mL, that were either stored refrigerated or under stress-temperature condition (40 °C) for 2 weeks. The samples were then analyzed by the SEC-HPLC method.

2.8. Covalent and non-covalent aggregates detection

The ability of the SEC-HPLC method to detect covalent and non-covalent aggregates of r-HuEPO was examined in this study. Heat induced r-HuEPO aggregate control stock solutions at 4 μg/mL concentration were prepared and analyzed as follows: (1) untreated, (2) with the addition of dithiothreitol (DTT) alone to reduce the disulfide bonds formed by thiol groups, (3) with the addition of guanidine HCl alone to solubilize non-covalent aggregates only and (4) with the addition of both DTT and guanidine HCl to reduce the disulfide bonds and to solubilize non-covalent aggregates. In steps 2, 3 and 4, disulfide bonds, which were reduced by DTT were then alkylated with iodoacetamide to prevent their reformation and excess iodoacetamide was neutralized with 2-mercaptoethanol.

Briefly, r-HuEPO aggregate samples at 4 μg/mL were prepared in phosphate buffer containing 0.015% polysorbate 80 to prevent non-specific adsorption. These samples were then incubated at room temperature (RT) for 1 h with DTT (50 mM) or with guanidine HCl (7.0 M) followed by DTT (1 h) in sequence. After the 1 h incubation with DTT, free thiol groups in the samples were alkylated by adding 2.0 M iodoacetamide to prevent reformation of disulfide bonds. Excess iodoacetamide was neutralized with 2-mercaptoethanol in all samples. The aggregates present in these samples were then quantified by SEC-HPLC.

2.9. Robustness determination

The robustness of the SEC-HPLC method performance was determined by testing for aggregate recovery after slightly varying the method conditions from optimized values. The parameters tested were variability in columns, column's temperature, the mobile phase pH, and isopropyl alcohol concentration in the mobile phase.

Table 1
Effect of 25% isopropyl alcohol in mobile phase on the stability of heat induced r-HuEPO aggregates

r-HuEPO purified bulk lot #	Percent (%) total r-HuEPO aggregate content by SEC-HPLC (mean, $n=3$)		% R.S.D. ($N=3$)
	With IPA in mobile phase/fluorescence detection	Without IPA in mobile phase/UV 280 nm detection	
757	54.14	53.11	1.9
759	52.71	52.58	1.9
784	52.25	53.11	1.6
785	50.96	50.98	1.4
786	54.50	54.56	1.9

Five batches of heat induced aggregates were tested by 2 SEC-HPLC methods using different detection systems and mobile phases (see Section 2.2). For each batch, the percent total aggregate (dimer and oligomer) content determined by both these methods was in close agreement (less than 2.0% R.S.D.) indicating that isopropyl alcohol in the mobile phase did not impact the separation or stability of aggregates in the SEC-HPLC method. SEC-HPLC, size exclusion high performance liquid chromatography; R.S.D., relative standard deviation; IPA, isopropyl alcohol.

3. Results

3.1. Optimization of isopropyl alcohol content in the mobile phase

Isopropyl alcohol was found to decrease the placebo interference (Fig. 1A) and the interfering peaks from placebo were completely absent when isopropyl alcohol was increased to 20–25% range in the mobile phase (Fig. 2A to E). To ensure reproducibility in the elimination of placebo peak interference the isopropyl alcohol concentration was maintained at 25% in the mobile phase.

3.2. r-HuEPO aggregate stability in the presence of 25% isopropyl alcohol

A close agreement (<2% R.S.D.) was observed between the results from the comparison of area percentages of heat induced r-HuEPO total aggregates in the presence and absence of 25% isopropyl alcohol in the mobile phase, indicating that the presence of 25% isopropyl alcohol had no impact on the r-HuEPO aggregates resolution or on the stability by SEC-HPLC method (Table 1).

3.3. Selectivity

Size exclusion chromatography was demonstrated to be selective and capable of resolving dimer and oligomer aggregate species of r-HuEPO from monomeric r-HuEPO and other excipients present in the formulation. When the mobile phase contained 25% isopropyl alcohol, no interfering peaks were present in the 18–27 min retention time range (Fig. 2D). All formulation placebo components, polysorbate 80, glycine, sodium chloride, and sodium phosphate eluted after 30 min, elute after 35 min and posed no interference to aggregate quantitation (data not shown).

3.4. Resolution of r-HuEPO aggregates from r-HuEPO monomer

The SEC-HPLC method was optimized to obtain the best resolution between r-HuEPO aggregates and monomer without interference from excipients. The average resolution parameters (mean ± R.S.D., $n=14$ independent runs, 4 μg/mL r-HuEPO) calculated by the US Pharmacopoeia method [20] between monomer and dimer was 0.95 ± 0.1, and between dimer and oligomer was 0.431 ± 0.2.

3.5. Linearity, recovery and precision

3.5.1. Assay linearity

The correlation coefficient (r^2) for r-HuEPO total aggregate control curves was found to be 0.995 or greater. Using the Waters 474 Fluorescence Detector, the typical slope value was 1.87×10^7 (fluorescence units) and the y-intercept was -0.014×10^{-7} fluorescence units. Using these control curves the LOD and LOQ values were determined to be 0.05 and 0.08 μg/mL, respectively. Compared to UV detection at 280 nm,

Table 2
Repeatability and intermediate precision for percent aggregate recovery from spiked r-HuEPO samples (inter-assay and intra-assay runs)

r-HuEPO concentration (μg/mL)	Dilution	% Aggregate spiked	Intra-assay variability						Inter-assay variability (%R.S.D., $n=9$)
			Day 1		Day 2		Day 3		
			Mean ($n=3$)	% R.S.D. ($n=3$)	Mean ($n=3$)	% R.S.D. ($n=3$)	Mean ($n=3$)	% R.S.D. ($n=3$)	
16.67	Undiluted	0.5	99.94	1.98	90.98	0.87	110.90	1.92	8.72
33.33	Undiluted	0.25	104.71	1.08	99.01	0.41	113.25	0.74	5.91
83.33	3-fold	0.2	95.43	2.68	93.52	1.87	107.64	1.55	6.94
333.33	6-fold	0.2	107.01	3.10	98.80	0.56	105.59	3.36	4.36

SEC-HPLC, size exclusion high performance liquid chromatography; R.S.D., relative standard deviation.

where r-HuEPO aggregates with concentrations less than or equal to 1.0 μg/mL are barely detectable, fluorescence detection had a sensitivity gain of over 10-fold.

3.5.2. Injection precision

The 6 injections of r-HuEPO (4.0 μg/mL) aggregate solution had the peak areas (mean ± % R.S.D. values) for oligomer, dimer, and monomer were 24.15 ($\times 10^6$) ± 1.0, 27.75 ($\times 10^6$) ± 1.1 and 25.79 ($\times 10^6$) ± 0.6, respectively. The peak area data generated had a percent R.S.D. value of 1.1 or less.

3.5.3. Assay recovery

The percent total aggregate recoveries for r-HuEPO samples in the 16.67–333.33 IU/mL concentration range, spiked with 0.2–0.5% of heat induced r-HuEPO aggregates (at LOQ levels), were within the 80–120% range (Table 2).

3.5.4. Precision

Repeatability, measured as the intra-assay variability (% R.S.D.) in aggregate recovery from spiked samples ($n = 3$ each on 3 days) was between 0.41 and 3.4% (Table 2).

3.5.4.1. Intermediate precision. Intermediate precision (or reproducibility), calculated the inter-assay variability (% R.S.D.) in aggregate recovery from spiked samples ($n = 9$ each on 3 days) was between 4.4 and 8.7% (Table 2).

3.6. Stability-indicating capability

The percent aggregate values measured ranged from less than LOQ to 0.09% for samples stored at 5 °C, and ranged between 0.82 and 1.61% for samples stressed at 40 °C for 2 weeks (Table 3). The data confirms that the method is stability-indicating and can detect changes in aggregates in samples that were stored under stress conditions compared to those samples stored refrigerated.

Table 3
Stability-indicating study: r-HuEPO aggregate content in samples stored at 5 and 40 °C (2 weeks)

r-HuEPO concentration (μg/mL)	% Total aggregates (mean, $n = 2$)			
	5 °C		40 °C	
	Sample 1	Sample 2	Sample 1	Sample 2
16.67	<0.21[a]	<0.21[a]	0.97	0.82
83.33	<0.08[a]	0.09[a]	1.42	1.36
83.33	<0.08[a]	<0.08[a]	1.61	1.54
333.33	<0.08[a]	<0.08[a]	1.13	1.22
333.33	<0.08[a]	<0.08[a]	0.98	1.10

[a] LOQ for 16.67 μg/mL is 0.21% and for 83.33 and 333.33 μg/mL is 0.08%.

3.7. Covalent and non-covalent aggregate detection

The results showed that the heat induced aggregate stock solution contained both covalent and non-covalent aggregates (Table 4). The untreated heat induced aggregates control contained 31.8% oligomer, 33.6% dimer aggregates and 34.6% monomer. Upon treatment with DTT (to reduce disulfide bonds) followed by alkylation, testing by SEC-HPLC resulted in area percentages for oligomer and dimer aggregates that were 19.5 and 14%, respectively. This indicated that 12.3% of the oligomer and 19.6% of the dimer aggregates were covalent, and were formed by disulfide bonds.

Similarly, the SEC-HPLC results of the heat induced aggregate stock solution treated with guanidine HCl alone (to solublize non-covalent complexes) showed a decrease of 8.0% in the oligomer content compared to the untreated r-HuEPO aggregates, suggesting the presence of non-covalent aggregates.

Table 4
Non-covalent and covalent r-HuEPO heat induced aggregate detection by the SEC-HPLC method

Sample description	Area percentages		
	Oligomer	Dimer	Monomer
Untreated r-HuEPO heat induced aggregates	31.8	33.6	34.6
DTT reduced and alkylated r-HuEPO heat induced aggregates	19.5	14.0	66.5
Guanidine HCl treated r-HuEPO heat induced aggregates	23.8	37.4	38.7
Guanidine HCl and DTT treated and alkylated r-HuEPO heat induced aggregates	8.4	21.8	69.8

DTT: dithiotretol; HCL: hydrogen chloride; r-HuEPO: recombinant human erythropoietin; SEC-HPLC: size exclusion high performance liquid chromatography.

Table 5
Robustness testing

Sample ID (μg/mL, % aggregate spiked)	Temperature			pH				Isopropyl alcohol content			
	25 °C	30 °C	% R.S.D. ($n = 2$)	pH 6.6	pH 6.8	pH 7.0	% R.S.D. ($n = 3$)	24%	25%	26%	% R.S.D. ($n = 3$)
16.67, 0.5% aggregate	111.4	109.2	1.4	113.4	111.4	93.8	1.3	89.1	104.5	107.3	9.8
33.33, 0.25% aggregate	87.8	107.5	14.2	107.9	87.8	96.3	14.5	107.1	98.9	96.6	5.5
83.33, 0.2% aggregate	91.1	95.4	3.3	94.7	91.1	98.9	2.8	93.1	90.3	86.9	3.4
333.33, 0.2% aggregate	98.8	99.3	0.4	103.6	98.8	91.5	3.4	99.8	102.1	107.5	3.8

Percent total aggregate recovery estimated by SEC-HPLC. Parameters tested: temperature, pH and isopropyl alcohol content in mobile phase. C: Celsius; IU: international units; R.S.D.: relative standard deviation.

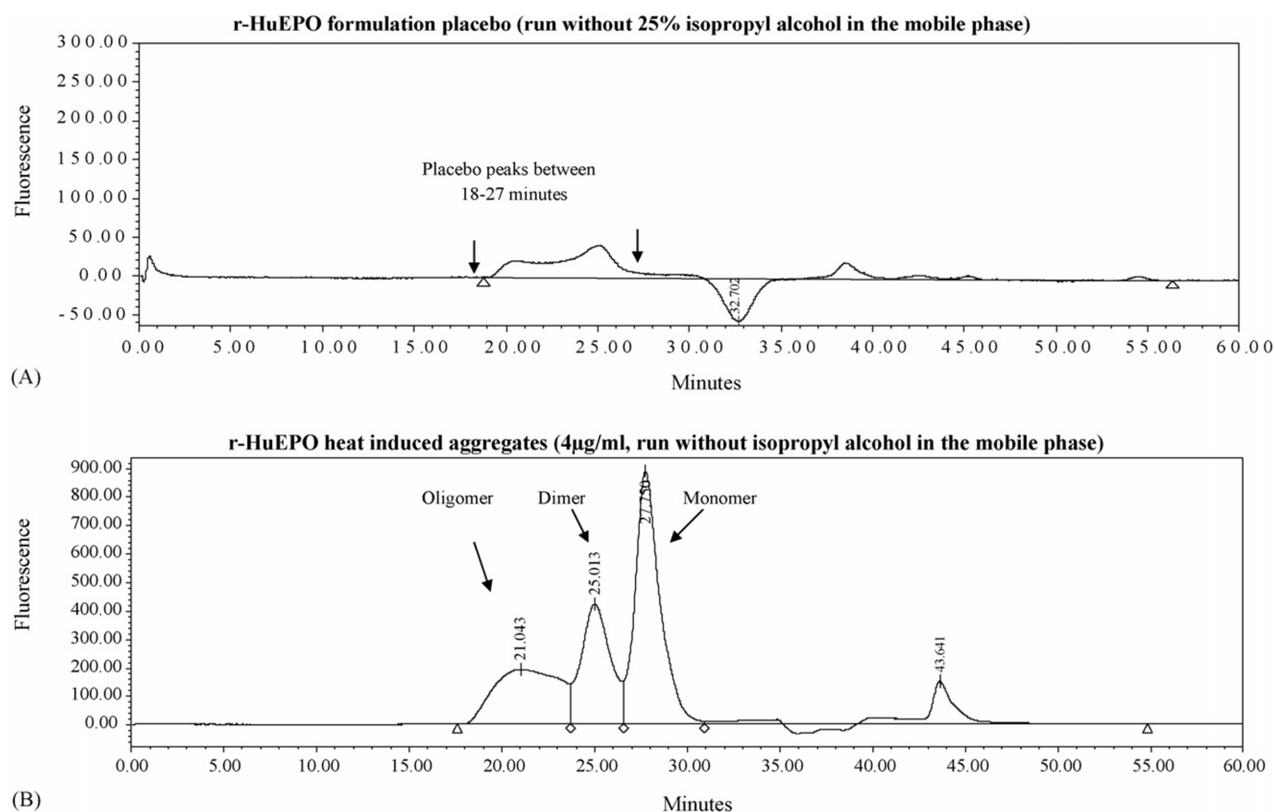

Fig. 1. Interference of formulation Placebo peaks between 18 and 27 min with r-HuEPO aggregate quantitation: recombinant human erythropoietin formulation placebo (no r-HuEPO present) was tested under SEC-HPLC method conditions without isopropyl alcohol in the mobile phase, and peaks from placebo appeared between 18 and 27 min of retention time (A). These peaks eluted in similar time range as that of the HuEPO oligomer, dimer and monomer (4 μg/mL) with approximate elution times of 21, 25, and 27 min, respectively (B).

The decreased oligomer content was recovered as dimers and monomers, which can be seen by corresponding increases in their area percentages (3.8 and 4.1%, respectively). When heat induced aggregates were treated with both guanidine HCl and DTT and were alkylated, only 8.4% oligomer was found; the rest being recovered as dimer or monomer. When compared to DTT treatment alone, approximately 11.1% more oligomer was further reduced to dimer or monomers with both guanidine HCl and DTT treatment, supporting that the oligomers involve non-covalent complexes.

3.8. Robustness

The robustness testing results indicated that the recovery of r-HuEPO aggregate spiked samples was between 80 and 120% range and did not further increase, supporting that the method's performance is robust enough to determine aggregate recovery within 20% variability (Table 5).

4. Discussion

Size exclusion chromatography is a routinely used analytical technique in quality control, which offers a convenient way to measure the dimer and oligomer aggregates of proteins present in test formulations [14]. This is a well-established technique, with the advantage that proteins up to 0.2 mg can be loaded on the column to detect very small amounts of aggregates in samples. The sensitivity attained by this technique is mainly dependent on the detection method used, such as UV, light scattering or fluorescence and the latter method offers enhanced selectivity and sensitivity for biopharmaceuticals [15]. Very few methods have been published to date, that provide a means of detecting very small amounts of r-HuEPO aggregates in formulated samples. Publications available in literature were mostly on the detection of unformulated r-HuEPO monomer in samples using various separation methods [16,17]. However, there are no published methods to quantitate r-HuEPO aggregates in pharmaceutical formulations with high sensitivity.

During the SEC-HPLC method development, it was observed that r-HuEPO formulation placebo components (no EPO present) eluted in the same retention time range (18–27 min) as that of the r-HuEPO dimer and oligomer aggregates (Fig. 1A and B) interfering with their quantitation. The co-eluting placebo component that interfered was identified as the excipient polysorbate 80 as a solution of polysorbate 80 in water alone resulted in interference similar to what was seen with formulation placebo. Polysorbate 80 was also found to shorten the life of the column, as determined by the loss of aggregate resolution and decreased performance over time.

Non-ionic surfactants, such as polysorbate 80, are commonly employed in many protein formulations, to prevent denaturation and formation of aggregates [12–15]. In addition to the formu-

lation, surfactants are used in sample diluent buffers in the range of 0.05–0.01% to prevent the non-specific adsorption losses of protein in vials and lab-ware when working at concentrations less than 1 μg/mL. Although there are no reports in literature to indicate that polysorbate 80 possesses intrinsic fluorescence properties our studies indicate that under the very high detector gain conditions employed in the method (1000 gain), fluorescence from polysorbate 80 was detected and interfered with the aggregate quantitation.

Among the approaches evaluated to decrease the placebo interference, the inclusion of 25% isopropyl alcohol in the mobile phase eliminated the co-elution of polysorbate 80 related peaks (Fig. 2E). The gradual increase of the isopropyl alcohol concentration from 5 to 25%, shifted the retention time of the placebo peaks between 18 and 27 min to beyond 28 min. It is hypothesized that the dissociation of these larger molecular weight placebo excipient components, into smaller components with an increase in the hydrophobic strength of the mobile phase could explain the above observed shift in the retention times to beyond 28 min and a decrease in fluorescence. Using 25% isopropyl alcohol in the mobile phase, r-HuEPO aggregate controls containing low concentrations of r-HuEPO aggregates in the range of 0.05–4.0 μg/mL, were analyzed in the presence of 0.03% polysorbate 80 without interference (Fig. 2E). The column life also improved with the use of 25% isopropyl alcohol in the mobile phase.

Because there were no commercially available r-HuEPO aggregate standards, heat induced r-HuEPO aggregates were used as standards for the purpose of r-HuEPO aggregate quantitation. The stability of the five lots of heat induced aggregate controls tested by two SEC-HPLC methods, both with UV detection (280 nm) and fluorescence detection, and both with and without 25% isopropyl alcohol in the mobile phases (Section 2.5), indicated that the heat induced r-HuEPO controls had similar compositions of total aggregates. These studies clearly confirmed that isopropyl alcohol, up to 25% concentration in mobile phase, had a negligible impact on the total aggregate composition (Table 1). Experiments performed with DTT and guanidine HCl on heat induced aggregates also indicate that at least 11% of the oligomer content is non-covalent and susceptible to guanidine HCl induced dissociation (Table 4). Interestingly, not all oligomer and dimer could be reduced to monomer, suggesting that the remaining 8.4% oligomers and 21.8% dimers could involve bonds other than disulfides or that they are not reducible by DTT. These results clearly demonstrate that the SEC-HPLC method is capable of detecting both covalent and non-covalent aggregates that are present in the heat induced r-HuEPO aggregates.

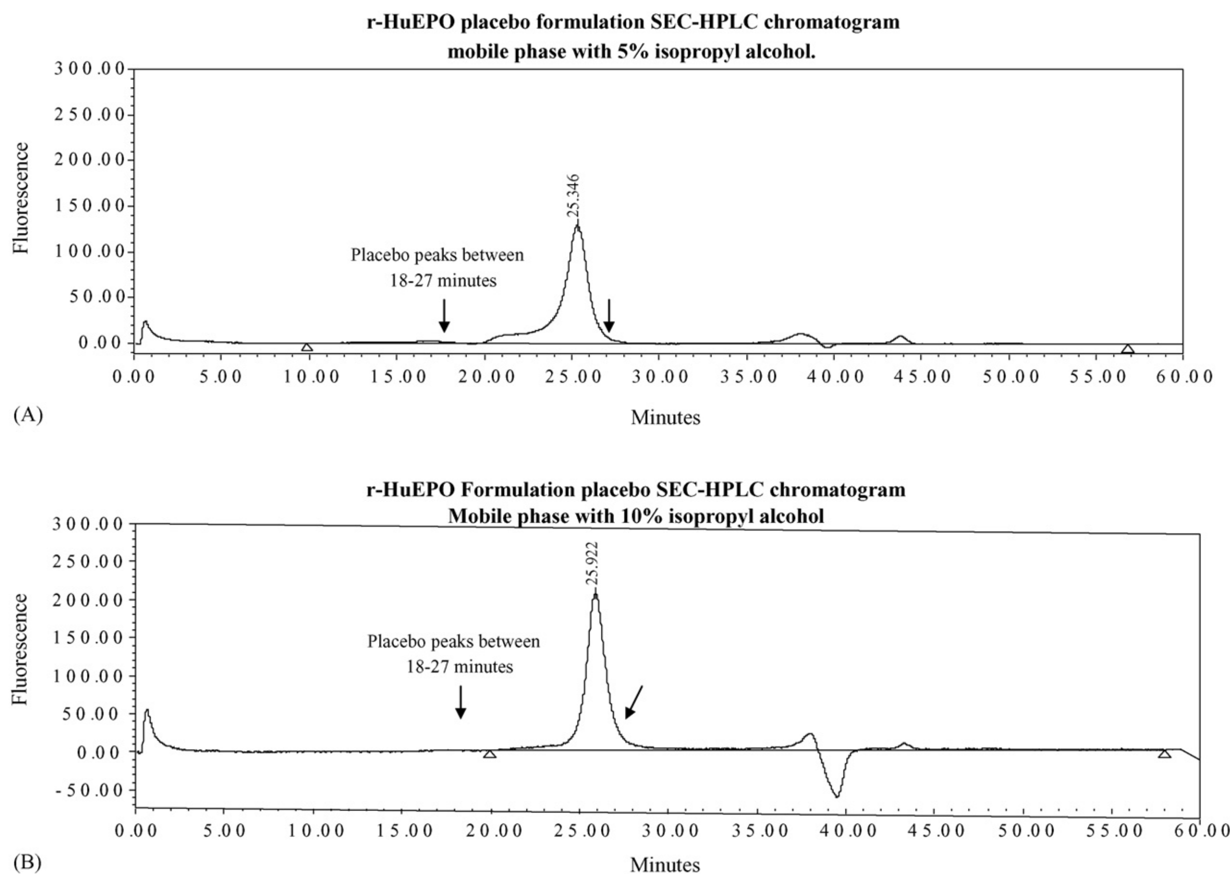

Fig. 2. Optimization of isopropyl alcohol content in mobile phase: the optimal amount of isopropyl alcohol for decreasing the placebo peaks was determined by varying its concentration from 5 to 25% (A–D) in mobile phase. Isopropyl alcohol, at 25% concentration, was found to eliminate the placebo peaks in the 18–27 min retention time range (D). E shows the elution profile of r-HuEPO aggregates (4 μg/mL), prepared in 0.03% polysorbate 80 and contained 25% isopropyl alcohol in the mobile phase.

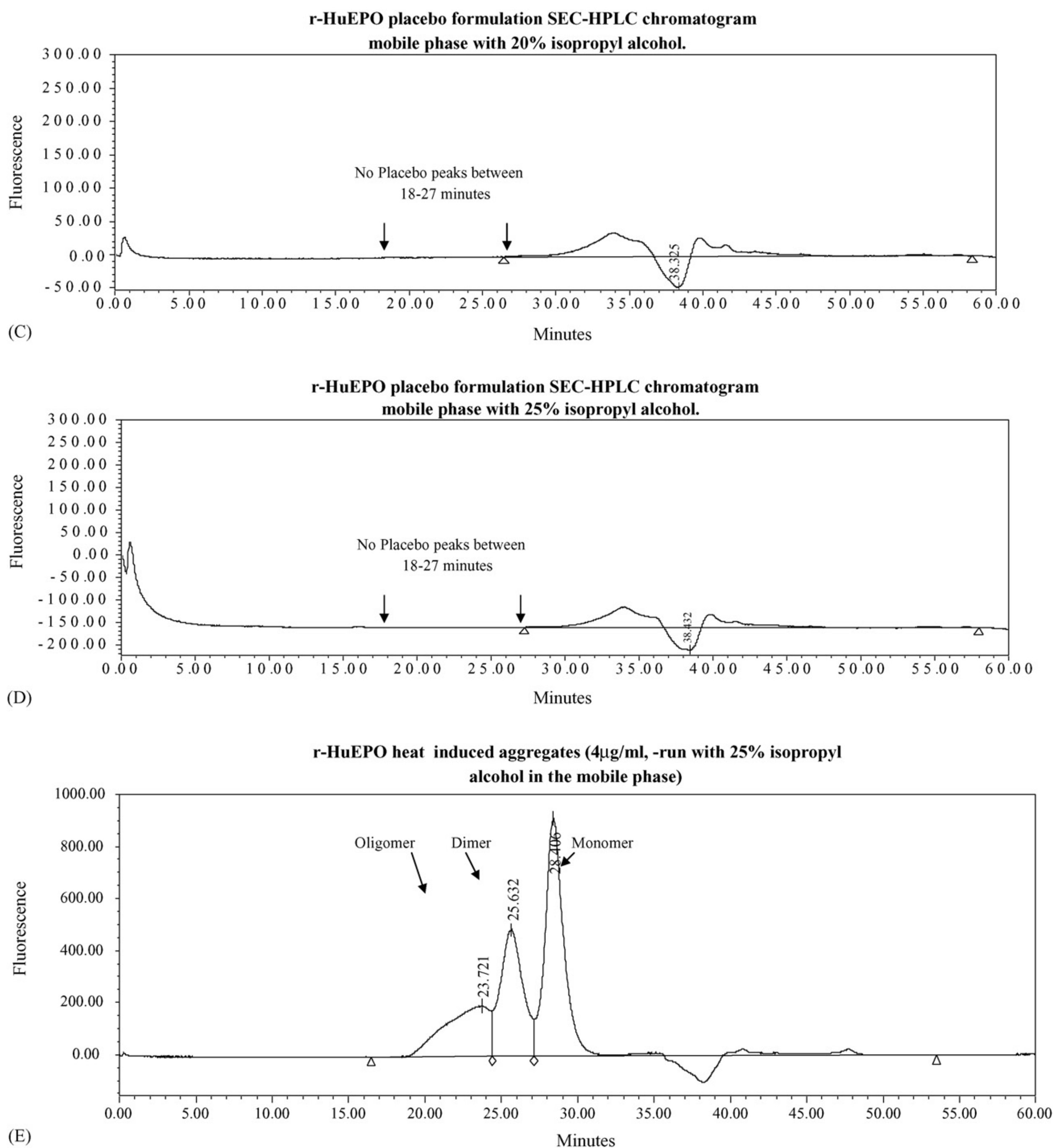

Fig. 2 (*Continued*).

The method measures very low r-HuEPO aggregate concentration (LOQ: 0.08 μg/mL) in presence of 200-fold higher protein concentration (16 μg/mL of r-HuEPO). Therefore, higher variability (20% R.S.D.) was observed during the aggregate recovery measurement of spiked samples prepared at this threshold LOQ. While the 80–120% recovery range (0.128–0.192 μg/mL) for a 1% (0.16 μg/mL) aggregate spiked r-HuEPO sample (at 16.7 μg/mL) might seem wide at the outset, this was observed at the LOQ levels and the method is still sensitive enough to monitor formulation stability for failing aggregates specifications (1%). Measurement of absolute recovery of the aggregates is not possible at these low concentrations, as the sample diluent buffer (containing 0.01% polysorbate 80) is necessary to keep the non-specific adsorption losses to a minimum.

The data demonstrated that the SEC-HPLC method has reliable precision, recovery, linearity, and selectivity for quantitation of the r-HuEPO aggregates in the presence of r-HuEPO monomers at various concentrations and with placebo components (Tables 2–4). The validated method is very sensitive and robust (at 20% R.S.D. level) and is suitable for the quantitation of r-HuEPO total aggregates in 0.03% polysorbate 80

containing formulated product with LOD and LOQ levels of 0.05 and 0.08 μg/mL, respectively. The finalized method is stability-indicating and is suitable for determining r-HuEPO total aggregates at 0.5, 0.25, 0.2 and 0.2% levels in the formulated product of r-HuEPO at concentrations of 16.67, 33.33, 83.33 and 333.33 μg/mL strengths, respectively. The SEC-HPLC method offers a robust way to measure total aggregates on a routine basis with a high sensitivity for use in product quality control.

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