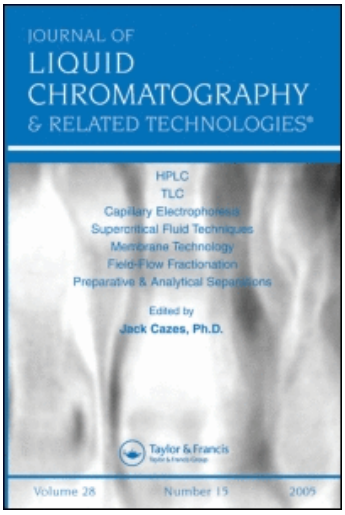

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Validation of a Size-Exclusion LC Method and Assessment of rhEPO in Pharmaceutical Formulations by Liquid Chromatography and Biological Assay

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Abstract: A size exclusion liquid chromatography (SE-LC) method was validated for the determination of erythropoietin in pharmaceutical formulations without serum albumin. The LC method was carried out on a BioSep-SEC-S 2000 column (300 mm × 7.8 mm I.D.) using photodiode array (PDA) detection at 214 nm. The mobile phase consisted of 0.001 M monobasic potassium phosphate, 0.008 M dibasic sodium phosphate, and 0.2 M sodium chloride buffer, pH 7.4, run isocratically at a flow rate of 0.5 mL/min. The chromatographic separation was obtained with retention time of 14.5 min, and was linear in the range of 5–150 μg/mL ($r^2 = 0.9991$). The accuracy was 101.07% with bias lower than 1.36%. The limits of detection and quantitation were 0.28 and 1 μg/mL, respectively. Moreover, method validation demonstrated acceptable results for precision and robustness. The proposed method was applied for the analysis of the erythropoietin in pharmaceutical dosage forms, and the content/potencies correlated to the bioassay, contributing to establish alternatives which improve the quality control, assuring the therapeutic efficacy.

Keywords: Erythropoietin, Pharmaceutical formulations, Size exclusion liquid chromatography, Validation

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INTRODUCTION

Erythropoietin (EPO) is an endogenous glycoprotein that is produced primarily by the interstitial cells in the kidneys, and is a main hormone involved in regulating red cells production. The human erythropoietin produced by recombinant technology (rhEPO) is now marketed worldwide with increasing clinical use for the treatment of anemia associated with chronic renal failure, cancer chemotherapy, and AIDS.[1–3]

The EPO molecule consists of a 165 amino acids polypeptide chain, heavily glycosilated at three N-linked and one O-linked glycosilation sites with two disulfide bonds, yielding a molecular mass of 34 kDa, and the carbohydrates comprising 40% of the weight. These play an important role in determining the biological activity of EPO, which appears to be dependent upon the number of sialic acid residues at the termini of the tri- and tetra-antennary sugar chains.[4–6]

The bioassays are useful to assess the quality, safety, and efficacy of those proteins, which can not be adequately characterized only by physicochemical tests. At the moment, the bioactivity of rhEPO has been determined by the normocythaemic and polycythaemic mice bioassays.[7–12] Today, many analytical techniques are available to monitor the purity, the chemical stability, and the potency of pharmaceutical proteins obtained through recombinant technology, but no single technique can satisfactorily provide sufficient information about a protein. Thus, a combination of physicochemical, immunological, and biological methods is recommended for the characterization and to monitor protein instability.[13,14]

The development of analytical methods for the direct analysis of rhEPO in pharmaceutical preparations present some difficulties, due to the low dose of the microheterogeneous glycoprotein in the presence of relatively large amounts of excipients, added to prevent adsorptions of the proteins to the vial walls and to increase stability during storage. Particular difficulties arise when the excipients are also proteins, such as human serum albumin.[15,16] The reversed phase liquid chromatography (RP-LC) exploits the hydrophobic properties of the molecules in the separation process and offers a high level of accuracy and sensitivity. A RP-LC method was validated for the analysis of rhEPO in pharmaceutical formulations, compared to the bioassay, and demonstrating it to be able to detect, separate, and quantify oxidized and deamidated proteins.[17] The EPO molecule 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 molecular weight aggregates can be formed and may have no, or reduced, activity and altered immunogenicity.[13] The stability of the protein can be monitored by size exclusion liquid chromatography (SE-LC), which resolves the possible dimers and aggregates.

A stability indicating SE-LC method with fluorescence detection and isocratic elution with isopropyl-alcohol in the mobile phase was applied for the quantitation of rhEPO aggregates in formulated products containing 0.03% polysorbate 80, with high sensitivity and robustness, and the run time of 60 min.[18] A high performance anion exchange chromatography method combined with intrinsic fluorescence detection was also developed for the determination of rhEPO in pharmaceutical preparations, showing a difference of 12% higher related to the claimed potency.[19]

The aim of the present study was to validate a sensitive and specific SE-LC method that can be used in combination with the RP-LC for the analysis of the rhEPO in pharmaceutical formulations, without serum albumin; moreover, evaluate the correlations between the physicochemical methods and the biological assay, contributing to improve the quality control, and to assure the therapeutic efficacy of this biotherapeutic.

EXPERIMENTAL

Chemicals and Reagents

European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP) for erythropoietin (250 μg/32,500 IU/vial), of a mixture of equal amounts of alpha and beta epoetins, was obtained from the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). A total of four batches of Eprex® (Roche, Rio de Janeiro, Brazil), containing 3,000 IU/0.3 mL (84 μg/mL) of erythropoietin were identified by Arabic numbers from 1 to 4, and four batches containing 10,000 IU/0.6 mL (138.33 μg/mL) were identified by Arabic numbers 5 to 8. The samples were obtained from commercial sources and used within their shelf life period. Monobasic potassium phosphate, dibasic sodium phosphate, sodium chloride, HPLC grade acetonitrile, sodium hydroxide, sodium EDTA, and trifluoracetic acid were obtained from Merck (Darmstadt, Germany). For all the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA) and reagents for automated counting were from HORIBA ABX Diagnostics (Montpellier, France). All other reagents were of the highest purity available from commercial sources. All chemicals used were of HPLC grade or special analytical grade.

Normocythaemic Mice Bioassay

The assay was carried out as previously published.[11] Female 8 week old BALB/c mice weighing between 18 and 23 g were allocated to sample,

standard, and control groups, in a fully randomized order and identified by colour code for the assay, with usually 6 mice per treatment group. The Ph. Eur. BRP for erythropoietin and test samples were diluted to the concentrations of 4, 12, and 36 IU per mL, with phosphate buffered saline (pH 7.2) containing 0.1% bovine serum albumin. Multiple injections of 0.2 mL rhEPO per mice were injected subcutaneously from day 1 to day 4. On day 5, peripheral blood was collected. Reticulocytes were counted by the automated flow cytometry method and the results reported as the percentage of reticulocytes. Statistical analyses of the assay data were carried out according to Finney, by parallel line methods (3×3), using PLA 2.0 software (Stegmann System-beratung, Rodgau, Germany).

Apparatus and Chromatographic Conditions

A Shimadzu LC system (Shimadzu, Kyoto, Japan) was used equipped with an SCL-10A_{VP} system controller, LC-10 AD_{VP} pump, DGU-14A degasser, CTO-10A_{VP} column oven, SIL-10AD_{VP} autosampler, and an SPD-M10A_{VP} photodiode array (PDA) detector. The detector was set at 214 nm for size exclusion and 280 nm for reverse phase, and peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.14 software program.

Reversed-Phase Chromatography (RP-LC)

The assay was carried out as described elsewhere.[17] The experiments were carried out on a reversed phase Phenomenex (Torrance, USA) Jupter C_4 column (250 mm × 4.6 mm I.D., with a pore size of 300 Å) and C_4 Kit Security Guard Cartridges were used to protect the analytical column. The LC system was operated at controlled ambient temperature (25°C) and the refrigerated autosampler maintained at approximately 5°C. The elution was performed by a gradient at a constant flow rate of 0.5 mL/min. Mobile phase A consisted of 0.1% trifluoracetic acid (TFA) and mobile phase B consisted of 0.08% TFA:acetonitrile (30:70, v/v). The gradient was linear with 0 to 100% of B from 0.1–60 min, and then re-equilibrated with mobile phase A during 15 min. The mobile phases were filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA). The injection volume was 50 μL for both standard and sample.

Size-Exclusion Chromatography (SE-LC)

The experiments were performed on a size exclusion Phenomenex (Torrance, USA) BioSep-SEC-S 2000 column (300 mm × 7.8 mm I.D.). A security guard holder was used to protect the analytical column. The

Shimadzu LC system was operated isocratically at ambient controlled temperature (25°C), using a phosphate buffered saline mobile phase consisting of 0.001 M monobasic potassium phosphate, 0.008 M dibasic sodium phosphate, and 0.2 M sodium chloride buffer, pH 7.4, and using PDA detection at 214 nm. This was filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA), and run at a flow rate of 0.5 mL/min. The temperature of the autosampler was kept at 5°C and the injection volume was 50 μL for both standard and samples.

Preparation of Samples and Standard Solutions

Working standard and sample solutions of rhEPO were prepared daily by diluting the Ph. Eur. BRP for erythropoietin and the samples of pharmaceutical formulation in mobile phase, to a final concentration of 33.6 μg/mL for the RP-LC method and 25 μg/mL for the SE-LC method, and in the phosphate buffered saline containing 0.1% bovine serum albumin, to appropriate concentrations for the bioassay.

Validation of the SE-LC Method

Once the chromatographic and the experimental conditions were optimized, the method was validated by the determination of the following parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability test, following the International Conference on Harmonisation (ICH) guidelines.[20]

Specificity

Specificity of the method towards the drug was established through the determination of the peak purity of samples of pharmaceutical formulation of erythropoietin (25 μg/mL) subjected to degradation, by maintaining the sample in water bath at 65°C during 6 h. Also, the interference of the excipients of the pharmaceutical formulations was analyzed by the injection of a sample containing only the placebo (in-house mixture of all the pharmaceutical formulation excipients). Then, the specificity of the method was established by determining the peak purity of erythropoietin in the samples using a PDA detector.

Linearity

Linearity was determined by constructing three independent analytical curves, each one with eight reference substance concentrations of the

Ph. Eur. BRP for erythropoietin, in the range of 5–150 μg/mL prepared in mobile phase. Before injection of the solutions, the column was equilibrated for at least 20 min with the mobile phase flowing through the system. Three replicates of 50 μL injections of the reference solutions were made to verify the repeatability of the detector response. The peak areas of the chromatograms were plotted against the respective concentrations of Ph. Eur. BRP for erythropoietin to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate calibration equation and determination coefficient.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was examined by six evaluations of the same concentration sample of rhEPO, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). Intra- and inter-day precision were expressed as relative standard deviation (RSD).

Accuracy

The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the formulations excipients with known amounts of the reference drug, to obtain solutions at concentrations of 20, 25, and 30 μg/mL, equivalent to 80, 100, and 120% of the nominal analytical concentration, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix and also expressed as the percentage relative error (bias %) between the measured mean concentrations and added concentrations.

Limits of Detection and Quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated, as defined by ICH,[20] using the mean values of three independent analytical curves, determined by a linear regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respectively, were multiplied by the ratio from the standard deviation of the intercept and the slope. The LOQ was also evaluated in an experimental assay.

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability for the routine analysis.[21,22] The robustness was determined by analyzing the same samples of rhEPO containing 25 μg/mL in triplicate by the one variable at a time approach, changing column temperature, injection volume, mobile phase pH, and flow rate. To assess the stability of sample solutions of erythropoietin, the samples were tested maintained at 2–8°C for 48 h, and also placed into the autosampler at 5°C, for 24 h. The stability of these solutions was studied by performing the experiment and observing any change in the chromatographic pattern compared with freshly prepared solutions.

System Suitability Test

To ensure the validity of the analytical procedure, data from five injections of 50 μL of the working standard solution containing 25 μg/mL were used for evaluation of the system suitability parameters, such as asymmetry, number of theoretical plates, retention time, and area, through the CLASS-VP® V 6.14 software.

Analysis of rhEPO in Pharmaceutical Formulations

For the quantitation of erythropoietin in the pharmaceutical formulations, eight batches containing, respectively, 84 or 138.33 μg/mL were diluted to appropriate concentration (25 μg/mL) with mobile phase, injected in triplicate, and the percentage recoveries of the drug calculated against the reference substance. The results were compared to those obtained using validated RP-LC and bioassay methods.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

To obtain the best chromatographic conditions, the mobile phase was optimized to provide appropriate selectivity and sensitivity. The use of phosphate buffered saline as mobile phase resulted in better sensitivity, compared with phosphate buffer and phosphoric acid, improving the peak symmetry (about 1.07) with the retention time suitable for the separation also of dimers and aggregate forms. For the selection of the best wavelength detection a PDA detector was used. The optimized

conditions of the LC method were validated for the analysis of rhEPO in pharmaceutical formulations due to the capability and application for the quality control.

Method Validation

Specificity

The analysis of the degraded forms showed the specificity of the analytical method. Typical chromatograms obtained with the resolution of the symmetrical peak corresponding to rhEPO, with the retention time of 14.5 min, were shown in Figure 1, demonstrating also that the proposed

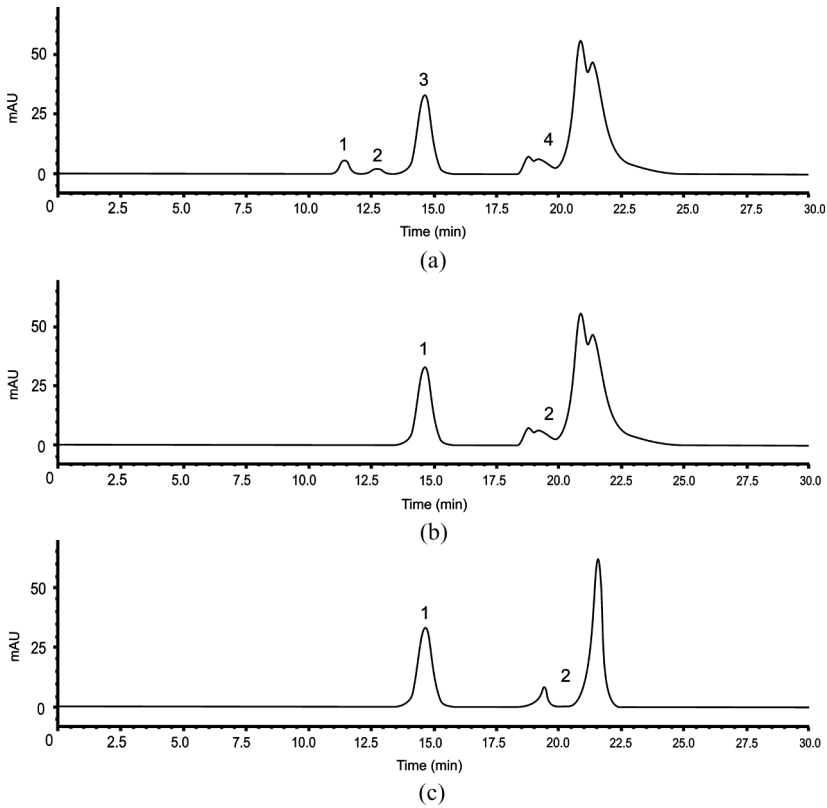

Figure 1. Representative SE-LC chromatograms of erythropoietin. (a) Pharmaceutical formulations, Peak 1: aggregates, peak 2: dimer, peak 3 monomer and peak 4: excipients. (b) Pharmaceutical formulations, Peak 1: monomer and peak 2: excipients. (c) Reference substance: Peak 1: monomer and peak 2: excipients.

method is able to detect and separate dimers, related substances of higher molecular mass, and the monomeric intact protein (Figure 1a). No interference from formulation excipients was found, showing that the peak was free from any coeluting peak, with values of peak purity index in the range of 0.999–1.000, thus confirming that the proposed method is specific for the analysis of erythropoietin.

Linearity

The analytical curves constructed for rhEPO were found to be linear in the 5–150 μg/mL range. The value of the determination coefficient calculated ($r^2 = 0.9991$, $y = (61422.53 \pm 94.82)x + (281740.55 \pm 5854.54)$, where, x is concentration and y is the peak absolute area) indicated the linearity of the analytical curve for the method.

Precision

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of 25 μg/mL performed on the same day and under the same experimental conditions. The RSD value obtained was 0.55%.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulations on three different days (inter-day); the mean values obtained were 103.04 and 102.95% with RSD 1.72 and 1.23%, respectively. Between analysts precision was determined by calculating the mean values and the RSD for the analysis of two samples of the pharmaceutical formulations by three analysts; the values were found to be 102.14 and 102.69% with RSD 1.36 and 1.06%, respectively. The results are shown in Table 1.

Accuracy

The accuracy was assessed from three replicate determinations of three different solutions containing 20, 25 and 30 μg/mL. The absolute means of obtained erythropoietin are shown in Table 2, with a mean value of 101.07% and bias lower than 1.36%, demonstrating that the method is accurate within the desired range.

Limits of Detection and Quantitation

For the calculation of the LOD and LOQ, a calibration equation, $y = 61422.53x + 281740.55$, was generated by using the mean values of the three independent analytical curves. The LOD and LOQ were

Table 1. Inter-day and between-analysts precision data of SE-LC for rhEPO in pharmaceutical formulations

	Inter-day			Between-analysts		
Sample	Day	Concentration found[a] (%)	RSD[b] (%)	Analysts	Concentration found[a] (%)	RSD[b] (%)
1	1	102.33		A	101.98	
	2	105.06	1.72	B	103.61	1.36
	3	101.73		C	100.84	
2	1	102.02		A	102.84	
	2	104.40	1.23	B	103.70	1.06
	3	102.44		C	101.54	

[a]Mean of three replicates.
[b]RSD = Relative standard deviation.

obtained by using the mean of the slope, 61422.53 ± 94.82, and the standard deviation of the intercept of the independent curves, determined by a linear regression line as 5854.54. The LOD and LOQ calculated were 0.28 and 0.95 μg/mL, respectively. The LOQ evaluated in an experimental assay, with the precision lower than 5% and accuracy within ±5%, was found to be 1 μg/mL.

Robustness

The results and the experimental range of the selected variables evaluated in the robustness assessment are given in Table 3, together with the optimized values. There were no significant changes in the chromatographic pattern when the modifications were made in the experimental

Table 2. Accuracy of SE-LC for rhEPO in pharmaceutical formulations

Nominal concentration (μg/mL)	Mean concentration found[a] (μg/mL)	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
20	20.15	1.28	100.73	0.75
25	25.27	1.25	101.10	1.08
30	30.41	0.71	101.37	1.36

[a]Mean of three replicates.
[b]RSD = Relative standard deviation.
[c]Bias = [(Measured concentration − Nominal concentration)/Nominal concentration] × 100.

Table 3. Chromatographic conditions and range investigated during robustness testing

Variable	Range investigated	Erythropoietin[a] (%)	RSD[b] (%)	Optimized value
Flow rate (mL/min)	0.45	97.45	1.47	
	0.50	101.54	0.10	0.50
	0.55	99.72	0.91	
Injection volume (μL)	30	98.18	0.79	
	40	99.08	0.61	50
	50	101.46	0.32	
Mobile phase pH	7.2	97.00	1.01	
	7.4	101.31	0.75	7.4
	7.6	99.86	1.44	
Solution stability	Autosampler 24 h	101.11	0.57	–
	2–8°C 24 h	100.55	1.02	–
	2–8°C 48 h	100.18	0.30	–

[a]Mean of three replicates.
[b]RSD = Relative standard deviation.

conditions, thus showing the method to be robust. The stability of the sample solutions was studied and the data obtained showed the stability during 24 h in the autosampler and during 48 h when maintained at 2–8°C.

System Suitability

The system suitability test was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed, using five replicates injections of a reference substance solution containing 25 μg/mL of rhEPO. The RSD values calculated for the retention time, peak symmetry, and peak area were 0.09, 1.10, and 0.76%, respectively. The number of theoretical plates was about 6089.8, with RSD of 1.17%. The experimental results show that the parameters tested were within the acceptable range (RSD < 2.0%), indicating that the system is suitable for the analysis intended.

Method Application

The proposed method was applied for the determination of rhEPO in pharmaceutical formulations against the Ph. Eur. BRP giving results in accordance with the label claim amounts, between 100.50 and 101.97%

Table 4. Analysis of the rhEPO related proteins and aggregates forms in pharmaceutical dosage forms

	SE-LC[a]			RP-LC[a]	
Sample	Monomer (%)	Dimer (%)	HMM (%)	Main peak (%)	Deamidates/ sulphoxides (%)
1	100.54	0.05	0.21	111.21	0.24
2	101.75	0.16	0.18	113.59	0.10
3	101.59	0.00	0.12	109.56	0.35
4	100.54	0.12	0.35	112.54	0.74
5	101.23	0.00	0.56	113.26	0.23
6	100.50	0.18	0.00	111.23	0.41
7	101.97	0.12	0.10	112.48	0.11
8	100.86	0.04	0.31	113.65	0.29
Mean	101.12	0.08	0.23	112.19	0.31
SD[b]	0.59	0.07	0.17	1.43	0.20

[a]Mean of three replicates.
[b]SD = Standard deviation.

as shown in Table 4. The higher molecular aggregates and dimeric forms were calculated and expressed as percentage of the total area obtained in the respective chromatographic procedure, showing values lower than 0.56%, and demonstrating the quality of the pharmaceutical samples

Table 5. Determination of the rhEPO potency in pharmaceutical dosage forms

			Bioassay[a]	
Sample	SE-LC[a,b] (%)	RP-LC[a,b] (%)	Potency (%)	Confidence intervals (P = 0.95)
1	102.50	112.30	100.21	75–139
2	102.71	111.91	99.85	68–145
3	99.83	108.25	97.50	71–138
4	102.75	112.20	100.11	79–129
5	105.40	113.65	102.90	81–152
6	98.45	108.55	96.55	69–144
7	101.22	110.14	97.54	68–139
8	103.32	113.48	101.50	74–131
Mean	102.02	111.31	99.52	—
SD[c]	2.16	2.09	2.17	—

[a]Non-significant difference (P > 0.05).
[b]Mean of three replicates.
[c]SD = Standard deviation.

and the applicability of the method for the quality control laboratories. Moreover, the estimated potency was compared to the normocythaemic mice bioassay and to the RP-LC method, performed in parallel, showing mean difference, respectively of $2.50\% \pm 1.07$ higher, and $9.29\% \pm 0.73$ lower, as shown in Table 5. The correlation between the methods was calculated by the Pearson's correlation coefficient, showing significant correlation with the bioassay ($r = 0.9629$) and with the RP-LC ($r = 0.9422$), in accordance also with the previously published data.[17] A current concern in the administration of recombinant derived proteins, is that presence of rhEPO related contaminants can have undesirable side effects and usually may have no, or reduced, activity. Therefore, the proper quality controls were taken to ensure that the levels of such forms were accurately determined, according to the limits for pharmaceutical products.

CONCLUSION

The results of the validation studies show that the SE-LC method is sensitive with a LOQ of 1 μg/mL, accurate with a mean value of 101.07%, and possesses significant linearity ($r^2 = 0.9991$), and without any interference from the excipients. The separation was achieved with the retention time of 14.5 min, and the method has been successfully used for analysis of pharmaceutical formulations without albumin, improving the quality control with advantages of lower time consumption related to the *in vivo* biological assay. Moreover, based on the correlation between the SE-LC and the normocythaemic mice bioassay, the combination of the validated physicochemical techniques employed offered a high degree of resolving power and selectivity, and is suggested as an alternative in the context of the 3R's, contributing to improve the quality control of rhEPO in pharmaceutical dosage forms.

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