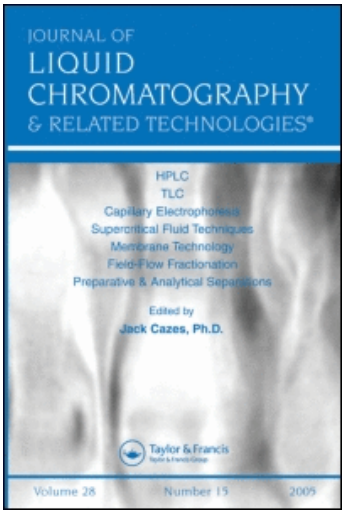

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Assessment of rhEPO in Pharmaceutical Formulations by a Reversed-Phase Liquid Chromatography Method and Bioassay

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Abstract: A gradient reversed-phase liquid chromatography (RP-LC) was developed for the analysis of alpha and beta rhEPO in pharmaceutical formulations. The RP-LC method was carried out on a Jupiter C_4 column (250 mm × 4.6 mm I.D., with a pore size of 300 Å). The elution was performed by a gradient at a constant flow rate of 0.5 mL/min. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) and mobile phase B consisted of 0.08% TFA:acetonitrile (30:70, v/v), using a photodiode array (PDA) detection at 280 nm. The chromatographic separation was obtained within 60 min and was linear in the concentration range of 10–150 μg/mL. The parameters validated, such as the specificity, precision, accuracy, and robustness gave results within the acceptable range. The pharmaceutical samples were analysed by the chromatographic method and compared to the normocythaemic mice bioassay, showing the mean difference between the estimated potencies of 11.2% ± 1.8 higher for the RP-LC, with significant correlation ($r = 0.9799$) as calculated by the Pearson's coefficient. The proposed RP-LC method represents an alternative to the bioassay that can be applied for the potency assessment, improving the quality control of rhEPO in pharmaceutical dosage forms.

Keywords: Recombinant human erythropoietin (rhEPO), Normocythaemic mice bioassay, Reversed-phase liquid chromatography, Validation

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INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by the kidneys, which regulates the production of the red blood cells in mammals. The erythropoietin consists of a 165 amino acids polypeptide chain (p*I* 4.4–5.1), heavily glycosilated at three N-linked and one O-linked glycosilation sites with two dissulfide bonds, yielding a molecular mass of 30–34 kDa. About 40% of the fully glycosilated EPO molecule consists of carbohydrate. 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.[1,2]

Since its cloning and subsequent recombinant production on an industrial scale, recombinant human erythropoietin (rhEPO) has become one of the most successful biopharmaceutical products. Therapeutically, rhEPO has proven beneficial for treating renal anaemia as well as anaemia of other chronic disorders, including autoimmune diseases, malignancies, and AIDS.[3–5]

Currently the content/potency of rhEPO preparations is tested by *in vivo* bioassays, which measure relevant biological activity. The assay carried out using the polycythaemic mice is based on the stimulation of reticulocyte production and ^{59}Fe incorporation into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.[6–8] Otherwise, the normocythaemic mice bioassay has been performed in normal animals using single or multiple injection protocols and the reticulocytes counted by flow cytometry.[9–11]

There have been decades of research on biologicals to reach a stage where the biological activity of some proteins could be correlated with physicochemical assays.[12–14] The development of analytical methods for the direct analysis of rhEPO in pharmaceutical preparations may present some difficulties due to the low dose of the micro-heterogeneous glycoprotein in the presence of relatively large amounts of the 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 (HSA), which can not be considered chemically homogeneous.[15,16]

Liquid chromatography (LC) is a well established technique, which has been successfully used to monitor the purity, identity, chemical stability, and potency of biopharmaceuticals obtained through recombinant technology.[17–19] Size exclusion liquid chromatography (SE-LC) may be used for quantitation, but is usually applied to determine the native size of the protein and to reveal possible dimmers, oligomers, and aggregates. A SE-LC with fluorescence detection was applied for the quantitation of rhEPO aggregates in formulated products containing 0.03% polysorbate 80, with high sensitivity and robustness.[20] A high performance anion exchange chromatography method combined with intrinsic fluorescence detection was also developed for the determination of recombinant human erythropoietin in pharmaceutical preparations, showing a difference of 12% higher than

claimed potency.[21] Besides, 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. The RP-LC method in combination with UV-detection, has been widely used for the quantitation of biological medicines, quality control, and for the analysis of closely related protein variants or degradation products, such as oxidised and deamidated forms.[19,22] At present, there is no published RP-LC method validated for the evaluation of rhEPO in pharmaceutical formulations.

The aim of the present study was to develop a RP-LC method validated by linearity, precision, accuracy, robustness, specificity, limit of detection, and limit of quantitation, to be applied for the potency assessment of rhEPO in pharmaceutical formulations; moreover, to evaluate the correlation between the chromatographic method and the biological assay, seeking for alternatives that can contribute to replace the bioassay and to improve the quality control of the biological medicine.

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 ten batches of erythropoietin commercial formulations were obtained from different manufacturers, identified by arabic numbers: 1 to 7 alpha epoetin containing 2,000 IU/mL and 4,000 IU/mL corresponding to 16.8 and 33.6 μg/mL, respectively; samples 8 to 10 of beta epoetin containing 10,000 IU/0.6 mL, corresponding to 138.33 μg/mL. All preparations were within their shelf life period. Hydrogen peroxyde 30% in aqueous solution, HPLC grade acetonitrile, sodium hydroxide, potassium phosphate monobasic, sodium phosphate dibasic, sodium EDTA, sodium chloride, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). For all analyses, ultrapure water (Millipore, Bedford, MA, USA) filtered through a 0.22 μm membrane filter was used, and reagents for automated counting were from HORIBA ABX Diagnostics (Montpellier, France). All other reagents were of the highest purity available from commercial sources.

Laboratory Animals

Female 8 week-old BALB/c mice were housed in air conditioned, controlled conditions (room temperature 22 ± 2ºC and relative humidity of 65%; artificial illumination, 12 hours per day) and used weighing between 18 and 23 g. They were given food and water ad libitum.

Normocythaemic Mice Bioassay

The assay was carried out as previously published by Ramos et al. 2003.[10] The animals were allocated to sample, standard, and control groups in a fully randomised 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 Analysis

Statistical analyses of the assay data were carried out according to Finney,[23] by parallel line methods (3×3), using PLA 1.2 software (Stegmann Systemberatung, Rodgau, Germany). Analysis of variance was performed for each assay and the assumption of linearity and parallelism of the log dose-log response lines was tested. Statistical weights were computed as the reciprocal of the variance of the log potency. Estimates of log potency were examined for heterogeneity using a χ^2 test, and were combined as weighted geometric means of homogeneous estimates. The outlier data were calculated by Dixon's test using the PLA 1.2. All the assays were analysed at a significance level of $P = 0.05$.

Apparatus and Reversed-Phase Liquid Chromatography 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 280 nm and peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.12 software program.

The experiments were carried out on a reversed phase Phenomenex (Torrance, USA) Jupiter C_4 column (250 mm $\times$ 4.6 mm I.D., with a pore size of 300 Å) and C_4 Kit Security Guard Cartridges was 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% trifluoroacetic 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 samples.

Procedure

Samples and Standard Solutions

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

Validation of the RP-LC Method

Once the chromatographic and the experimental conditions were optimized, the method was validated by the determination of the following parameters: specificity, linearity, range, precision, accuracy, robustness, limit of detection (LOD), and limit of quantitation (LOQ), following the ICH guidelines.[24]

Specificity

Specificity of the method towards the drug was established through the determination of the peak purity of samples of pharmaceutical formulation of rhEPO (33.6 μg/mL) subjected to degradation by oxidative conditions, by adding 30 μL of hydrogen peroxide 30% during 1 and 2 hours. Besides, the in-house mixture of the pharmaceutical formulations, excipients were also analysed by the RP-LC method using a PDA detector.

Linearity

The range of linearity was determined by constructing three calibration curves. For the construction of each calibration curve, seven concentrations of the Ph. Eur. BRP for erythropoietin in the range of 10–150 μg/mL were prepared. Before injection of the solutions, the column was equilibrated for at least 30 min with the mobile phase following through the system. Each measurement was carried out in three replicates of 50 μL injection for the standard solution, to verify the repeatability of the detector response at each concentration. The peak areas of the chromatograms were plotted against the concentrations of Ph. Eur. BRP for erythropoietin to obtain the calibration

curve. The seven concentrations of the standard solution were subjected to regression analysis to calculate calibration equations and correlation coefficients.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was determined by performing six repeated analysis of the same samples 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-day) and also by another analyst performing the analysis in the same laboratory (between-analysts).

Accuracy

The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the formulation excipients with known amounts of the reference drug, to obtain solutions at concentrations of 26.88, 33.60, and 40.32 μg/mL equivalent to 80, 100, and 120%, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

Robustness

The robustness was assessed by altering the following experimental conditions such as: by changing the flow rate from 0.45 to 0.55 mL/min, the injection volume from 30 to 50 μL, the wavelength in the range of 210 to 320 nm, the percent of acetonitrile from 68 to 72%, and stability of the analytical solution in the autosampler maintained at approximately 5°C.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For the calculation of the LOD and the LOQ, a calibration equation was generated by using the mean values of the three independent calibration curves. The LOD and the LOQ were obtained by using the mean of the slope, and the standard deviation of the intercept of the independent curves, determined by a linear regression line as defined by ICH.[24]

System Suitability

To ensure the validity of the analytical procedure, data from five injections of 50 μL of the working standard solution containing 33.6 μg/mL were used for the evaluation of the system suitability parameters, such as asymmetry,

number of theoretical plates, retention time, and area, through the CLASS-VP® V 6.12 software.

RESULTS AND DISCUSSION

Validation of the RP-LC Method

A typical chromatogram was obtained by the proposed RP-LC method, demonstrating the resolution of the symmetrical peak corresponding to rhEPO with a retention time of 49.9 min, and no interfering peaks due to the excipients are shown in Figure 1. The specificity of the analytical method was indicated by the oxidative conditions that generated no additional peaks. The studies with the PDA detector showed that the rhEPO peak was free from any coeluting peak, thus demonstrating that the proposed method is specific for the analysis of rhEPO.

The calibration curves for rhEPO were constructed by plotting the area of the peaks versus concentration. Linearity was observed in the concentration range from 10 to 150 μg/mL. The value of the determination coefficient ($r^2 = 0.9997$, $y = 3744.1 \pm 135.1x - 11.695 \pm 4687.45$) indicated significant linearity of the calibration curve for the method.

The precision of an analytical method was studied as the repeatability by calculating the relative standard deviation (RSD) for six determinations of the concentration of 33.6 μg/mL. The mean of the determinations of rhEPO in the samples of pharmaceutical formulation was 100.06%, with the RSD calculated as 0.70%.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day, Table 1); the RSD values obtained were 1.27 and 1.04%, respectively. Between analysts, precision was determined by calculating the RSD for the analysis of two samples of the pharmaceutical formulation by two analysts, and the values were found to be 0.46 and 0.25%, respectively (Table 2).

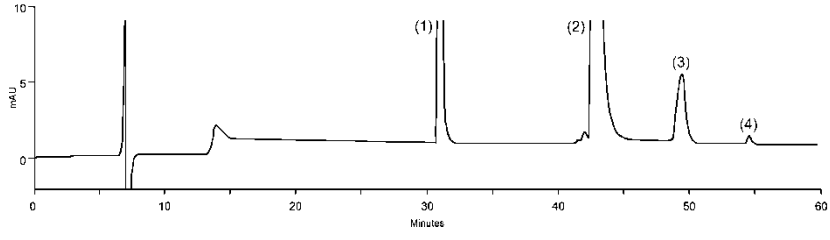

Figure 1. RP-LC chromatogram of rhEPO pharmaceutical formulation (33.6 μg/mL): peak 1, 2 and 4 – excipients, peak 3 – rhEPO.

Table 1. Inter-day precision data of RP-LC for rhEPO in samples of pharmaceutical formulation

Sample	Day	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
1	1	100.49	100.70	1.27
	2	102.08		
	3	99.54		
2	1	100.88	100.52	1.04
	2	99.34		
	3	101.34		

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

The accuracy was assessed from three replicate determinations of three different solutions containing 26.88, 33.60, and 40.32 μg/mL. The absolute means obtained were 99.81, 101.13, and 100.96%, respectively, with a mean value of 100.66% and RSD of 0.72% as shown in Table 3. It is evident that the method is accurate within the desired range.

The robustness was determined by analyzing the same pharmaceutical samples under the variety of conditions described. The results and the experimental range of the selected variables are given in Table 4, together with the optimized values. There were no significant changes in the chromatographic pattern when the above modifications were made in the experimental conditions, thus showing that the method is robust.

The LOD and the LOQ calculated were 3.75 and 12.51 μg/mL, respectively. The LOQ evaluated in an experimental assay, with the precision lower than 5% and accuracy within ±5%, was found to be 10 μg/mL.

The system suitability test was also carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed. Results of

Table 2. Between-analysts precision data of RP-LC for rhEPO in samples of pharmaceutical formulation

Sample	Analyst	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
1	A	100.49	100.82	0.46
	B	101.15		
2	A	100.64	100.46	0.25
	B	100.28		

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

Table 3. Accuracy of RP-LC for rhEPO

Nominal concentration (μg/mL)	Mean concentration found[a] (μg/mL)	RSD[b] (%)	Accuracy (%)
26.88	26.83		
33.60	33.98	0.72	100.66
40.32	40.71		

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

the system suitability test are given in Table 5, showing that the parameters are within the suitable range.

Analysis of Pharmaceutical Dosage Forms by Physicochemical and Biological Methods

The content/potency of the rhEPO pharmaceutical preparations usually range from 2,000 to 16,666 IU/mL, which is equivalent to 16.8–138.33 μg/mL, demonstrating that the linearity range evaluated from 10 to 150 μg/mL is sensitive enough to assay the licensed pharmaceutical products. The same ten commercial batches of alpha and beta rhEPO forms in pharmaceutical formulations containing HSA or polysorbate 20, were analysed in parallel by the RP-LC method and the normocythaemic mice bioassay against the Ph. Eur.

Table 4. Chromatographic conditions and range investigated during robustness testing

Variable	Range investigated	rhEPO[a] (%)	Optimized value
Flow rate (mL/min)	0.45	101.83	
	0.5	99.98	0.5
	0.55	99.64	
Injection volume (μL)	30	99.42	50
	40	99.74	
	50	100.12	
Acetonitrile (%)	68	100.65	
	70	99.97	70
	72	99.75	
Solution stability	Autosampler		
	24 h	99.7	—
	48 h	99.3	—
Wavelength (nm)	210–320	—	280

[a]Mean of three replicates.

Table 5. Results of the system suitability test

Parameter	Minimum	Maximum	RSD[a] (%)	Status
Asymmetry	1.03	1.05	0.87	Passed
Theoretical plates	42069	42718	0.77	Passed
Retention time	49.82	49.99	0.17	Passed
Area	135668	138031	0.86	Passed

[a]RSD = Relative standard deviation.

BRP for erythropoietin, giving the mean difference between estimated potencies of 11.2% $\pm$ 1.8 lower for the bioassay compared to the RP-LC, as shown in Table 6. The values obtained from the two methods were compared statistically by the Pearson's correlation coefficient, showing significant correlation (r = 0.9799). Therefore, the RP-LC method is suggested as an alternative to the bioassay of rhEPO in pharmaceutical formulations, in the context of the reduction and replacement of the animal models for the biological assay.

Table 6. Determination of the rhEPO potency in pharmaceutical formulations against the biological reference preparation for rhEPO

	Nominal concentration			Bioassay	
Sample	IU/mL	μg/mL	RP-LC[a] (%)	Potency (%)	Confidence intervals (P = 0.95)
1	2000	16.8	104.4	91.9	70–125
2	4000	33.6	121.4	108.1	84–147
3	4000	33.6	129.8	118.4	92–152
4	4000	33.6	121.7	108.4	84–149
5	4000	33.6	100.9	91.2	68–128
6	4000	33.6	115.1	105.7	74–139
7	4000	33.6	106.8	95	71–129
8	16666	138.33	112.9	103.9	81–144
9	16666	138.33	116.6	103.4	78–149
10	16666	138.33	107.2	98.3	72–139
Mean			113.6	102.4	
SDb			9.0	8.5	

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

CONCLUSION

The proposed RP-LC method was optimized and validated by the specificity, accuracy, and precision, demonstrating acceptable results. The separation was achieved with the retention time of 49.9 min, and the method has been successfully used for the analysis of commercial pharmaceutical formulations, with advantages, also, of lower time consumption related to the biological assay. Moreover, the RP-LC can represent an important alternative to the bioassay improving the existing procedures for the potency evaluation of rhEPO, which can be applied to the purification process and to the quality assessment of rhEPO biopharmaceutical products.

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