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ORIGINAL ARTICLE

Development and validation of RP-HPLC and RP-UPLC methods for quantification of erythropoietin formulated with human serum albumin

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Abstract Rapid and sensitive reverse phase high performance liquid chromatography (RP–HPLC) and ultra performance liquid chromatography (UPLC) methods with UV detection for quantification of erythropoietin (EPO) in presence of human serum albumin (HSA) as a stabilizer in a pharmaceutical formulation of EPO have been developed and validated. Chromatography was performed with mobile phase containing 0.1% Trifluoroacetic acid (TFA) in MilliQ water and 0.1% TFA in acetonitrile with gradient program and a flow rate of 1.5 mL/min for HPLC and 0.35 mL/min for UPLC. Quantification was accomplished with internal reference standard (qualified using EP reference standard). The methods were validated for linearity (correlation coefficient = 0.99), accuracy, precision and robustness. Robustness was confirmed by considering three factors; percentages of TFA in mobile phase, age of test sample and mobile phase and column temperature. Intermediate precision was confirmed by different analysts, different equipments and on different days. The relative standard deviation (RSD) value (<2%, $n=30$) indicated good precision of the developed method. The proposed RP-HPLC method had retention time less than 20 min while the developed UPLC method had retention time less than 4 min. Both the RP-HPLC and UPLC methods were simple, highly sensitive, precise and accurate, suggesting that the developed methods are useful for routine quality control.

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1. Introduction

Erythropoietin (EPO) is a glycoprotein produced primarily by the kidney, which regulates the production of red blood cells [1]. EPO has been expressed using recombinant DNA technology and pharmaceutical preparations of recombinant human EPO are now available from several manufacturers around the world [2].

Since recombinant protein molecule readily undergoes structural changes as a result of oxidation, deamination and aggregation (dimer and polymer formation), appropriate

formulation of a medicinal product ensuring its stability throughout the reported shelf life, is important [3–5].

The development of analytical methods for the direct analysis of the active drug substance (protein) in these pharmaceutical preparations may present some difficulties as the amount of EPO present in these formulations is very small compared to the large amount of HSA added as an excipient to prevent adsorption of the protein to the vial/storage container walls and to increase stability during storage [6,7]. Serge Rudaz et al. have summarized in their review the analytical techniques available for intact biopharmaceutical protein determination [8]. Although some EPO formulations in the market are formulated with low molecular mass excipients, some manufacturers use HSA as a stabilizer, in a quantity of 1–3 mg/mL which is 100 fold more by weight than the active substance content.

These formulated products require specific analytical methods to control the quality of the product. Gunturi et al. have developed a method for the determination of rHu EPO aggregates in formulations [9]. Among the possible methods to eliminate HSA, immunoaffinity chromatography (IAC) is one of the most effective ones [10]. However, there is no method reported for the determination of EPO in the presence of HSA without any sample pretreatment. Although HPLC methodologies have been described previously, they have been developed either for analysis of purified r-Hu EPO monomeric protein [11–13] or for investigation of r-Hu EPO (monomer) metabolic pathways [14] in the absence of HSA.

Capillary electrophoresis (CE) methods have been developed to characterize the EPO glycoform pattern and a capillary zone electrophoresis method has been described in the EPO monograph of European Pharmacopoeia (EP) as an identification test [15]. In addition to this method, another CE method has been developed that is capable of analysing EPO pharmaceutical preparations containing salts and HSA, in the concentration range of 0.03–1.92 mg/mL of EPO. Luykx et al. have developed a high-performance anion-exchange chromatography (HPAEC) method with intrinsic fluorescence detection for determination of rHu EPO in pharmaceutical products [16]. This method appears to be a suitable method for differentiating alpha and beta rHu EPO. Furthermore during the course of their work when they tried to compare HPAEC fluorescence detection method with HPAEC UV detection method, a low signal to noise ratio was obtained, which attributed to the presence of HPES (*N*-2- hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer in the eluent [16].

The objective of the study was to develop methods, using 'RP-HPLC and UPLC' techniques to enable quantification of EPO in medicinal formulations containing HSA.

This paper reports rapid and sensitive RP-HPLC and UPLC methods with UV detection, which are useful for routine quality control of EPO in different pharmaceutical formulations containing HSA. Both methods were validated by parameters such as linearity, accuracy, precision and robustness. To the best of our knowledge, no UPLC method has been reported for the assay of EPO in the presence of HSA.

2. Experimental

2.1. Materials, reagents and chemicals

HPLC grade acetonitrile was purchased from Merck; trifluoro-acetic acid was purchased from Sigma Aldrich. Ultra pure water was obtained using Milli-Q® UF-Plus (Millipore) system. HSA with 20% globulin fraction was obtained from Baxter. EPO internal reference standard (IRS) having 0.8 mg/mL concentration was used as standard, it was qualified using EP reference standard. Formulated EPO was used as samples. Other chemicals, such as tri-sodium dihydrate, sodium chloride and citric acid used, were of "highest purity" available.

2.2. Preparation of standard, mobile phase and dilution buffer

Diluted EPO standard was prepared by using 0.8 mg/mL of EPO IRS and it was used for preparation of different working standards using dilution buffer or dilution buffer containing 2.5 mg/mL of HSA. Mobile phase 'A' consisted of 0.1% (v/v) TFA in Milli Q water and mobile phase 'B' consisted of 0.1% (v/v) TFA in acetonitrile. Dilution buffer (Citrate buffer) containing 5.8 mg/mL tri-sodium dihydrate, 5.8 mg/mL sodium chloride and 0.06 mg/mL citric acid in "Milli Q water" was prepared and used so as to have a matrix similar to EPO formulation. Dilution buffer with HSA was prepared by diluting 2.5 mg/mL of HSA in dilution buffer. All dilutions were made using calibrated digital micro-pipettes.

2.3. Chromatographic condition

An LC system equipped with an injection valve (quaternary), 215 UV detector and chemstation software was used for RP-HPLC method. A reverse -phase C_8 column (4.6 mm ID × 250 mm L, porosity 300° A, particle size 5 μm) with guard column (reverse-phase C_{18} column of 4.6 mm ID × 35 mm L, porosity 300° A, particle size 5 μm) was used for separation. To get the optimum results, mobile phase with a flow rate of 1.5 mL/min and column temperature at 45 °C were used. The gradient programme for mobile phase was optimized using a timed gradient programme T (min)/%mobile phase A: 0/65, 4/65, 12/50, 14/50, 15/40, 16/65, 20/65.

An LC system equipped with an injection valve (binary), a 210 UV detector and Empower software was used for RP-UPLC method. Reverse-phase C_{18} column (2.1 mm ID × 50 mm L, porosity 135°A, particle size 1.7 μm) was used for separation. To get the optimum results, mobile phase flow rate was kept constant at 0.35 mL/min and column temperature was set at 60 °C. The gradient programme for mobile phase was optimized using a timed gradient programme T (min)/% mobile phase A: 0/85, 0.12/85, 0.33/70, 0.62/64, 2.62/35, 3.19/0, 3.76/85, and 4.05/85.

2.4. Validation of chromatographic methods

The optimized chromatographic methods were validated according to the procedures described in ICH guidelines Q2 (R1) [17]. The following validation characteristics were addressed: specificity, linearity and range, accuracy, precision, and robustness.

3. Results and discussion

3.1. RP-HPLC method

3.1.1. Method development

Initially, the gradient HPLC conditions were optimized for EPO in presence of HSA. The chromatographic separation was

achieved by applying chromatographic conditions described in Section 2.3.

The applied chromatographic conditions permitted a good separation of HSA and EPO at different concentrations of EPO each containing 2.5 mg/mL of HSA. No interference of HSA and other excipients was observed during the analysis as seen from Fig. 1.

The capacity factor (k') of first peak (HSA) and second peak (EPO) was 3.24 and 5.24, respectively; while the resolution factor was 6.88. The asymmetry of the peak was found to be 1.29 and 5.29 for EPO and HSA, respectively; while the tailing factor parameter was found to be 1.29 and 1.14 for EPO and HSA, respectively. For replicate injections of EPO standard the RSD of the main peak area was found to be below 0.7%, and there was no variation in the retention time (less than 0.1 min).

Based on the studied parameters, it was concluded that the EPO and HSA peaks were well resolved in the developed method and the tailing factor was within limits.

3.1.2. *Method validation*

3.1.2.1. Specificity. To evaluate possible interfering peaks, three different concentrations of EPO (0.04, 0.1 and 0.4 mg/mL) with excipients (HSA, tri-sodium dihydrate, sodium chloride and citric acid) were prepared in mobile phase and in formulation buffer. No interference was observed.

3.1.2.2. Linearity and range. EPO IRS was used for preparation of different concentrations of EPO ranging from 0.028 to 0.130 mg/mL, each containing 2.5 mg/mL HSA. Linearity curves were plotted for 0.04 and 0.1 mg/mL of EPO (Fig. 2). The correlation coefficient, slope, Y-intercept, regression equation of the calibration curve were determined and are shown in Table 1. The percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 97%–103%.

3.1.2.3. Accuracy. Accuracy was studied using two different sets of three different solutions, containing 0.032, 0.040 and 0.048 mg/mL and 0.08, 0.10 and 0.12 mg/mL of EPO. Each solution in its formulation buffer and in the mobile phase was spiked with HSA at a concentration of 2.5 mg/mL. The percent recovery was found to be more than 95% for 0.04 mg/mL of EPO while the percent recovery was found to be more than 99% for 0.1 mg/mL of EPO in presence of 2.5 mg/mL of HSA. The percent RSD was found to be less than 2.0%.

3.1.2.4. Precision. Precision was evaluated for inter-day (Repeatability) and intra-day (Intermediate precision) variation, and different makes of columns. The repeatability was assessed with six independent sample preparations for each of the three different system suitability samples (0.04 mg/mL, 0.1 mg/mL and 0.4 mg/mL of EPO) and single injection was injected from each preparation. The percent RSD of peak area, for each EPO IRS was found to be less than 0.9%. Intra-day precision was determined by a ten fold analysis of 0.04 mg/mL of EPO spiked with 2.5 mg/mL of HSA. The precision of the method was evaluated by performing five different conditions ($n=30$) and calculating the relative standard deviations (RSD). Three replicate injections of system suitability standards prepared independently were considered for the study. The percent RSD for the main peak area of EPO standard within each set and between different sets was found to be less than 2.0%. The percent recovery of each EPO standard was found to be between 95.0%–105.0% and the maximum variation between sets was found to be 5.0%.

3.1.2.5. Robustness. The robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its robustness during normal usage. Robustness was tested using three variables, age effect of mobile phase and test samples, column temperature and mobile phase composition.

3.1.2.6. Age effect of mobile phase and test samples held for seven days. Freshly prepared samples for system suitability (0.04, 0.1 and 0.4 mg/mL of EPO) and those prepared seven days ago were analyzed using both freshly prepared and seven day old mobile phase. There was not much variation in the results, with percent variation from initial day to 7 day being about 5% and percent RSD being less than 0.4%. There was no difference in retention time and percent recovery was found to be between 90% and 110%. It is thus recommended to use freshly prepared sample as well as mobile phase for analysis.

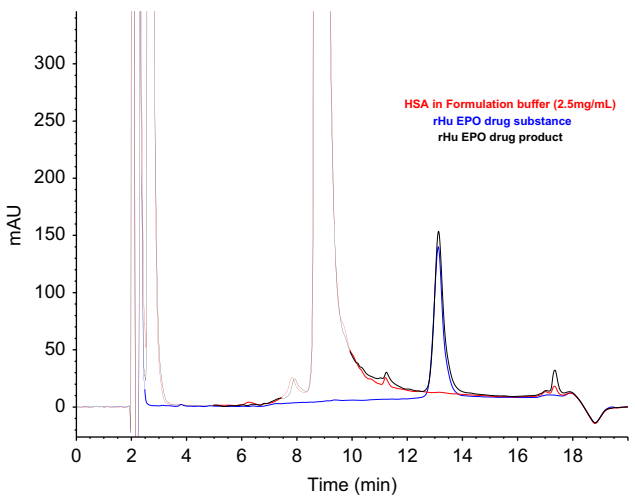

Figure 1 HPLC chromatograms of internal reference standard of EPO, HSA in formulation buffer (2.5 mg/mL) and EPO drug product.

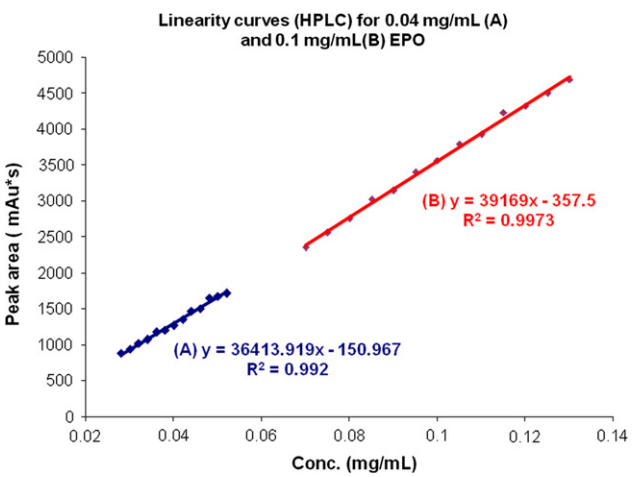

Figure 2 Linearity curves (HPLC) for 0.04 mg/mL (A) and 0.1 mg/mL (B) EPO.

Table 1 Results of regression equation/correlation coefficient and both methods comparative data.

Statistical parameter	HPLC for 0.04 mg/mL	HPLC for 0.1 mg/mL	UPLC
Linearity and range (Conc. in mg/mL)	0.028 to 0.052	0.07 to 0.13	0.0025 to 0.15
Regression equation	$y=36413.919x-150.967$	$y=39168.864x-357.502$	$y=10602431.0891x-725.9877$
Correlation coefficient (R^2)	0.992	0.997	0.999
Total analysis time (min)	20	20	4
Retention time (min)			
For HAS	9		1.3
For EPO	13		1.9
Sample size	100 µL		5 µL
Specificity	No interference		No interference
Accuracy	Recovery more than 95%		Recovery more than 97%
Precision	RSD less than 2%		RSD less than 1.5%
Robustness	Method is robust for all tested parameters except age effect of mobile phase and test samples		Method is robust for all tested parameters.

3.1.2.7. Column temperature effect. Experiments were conducted using system suitability samples with column temperature variation of +2 °C from the set temperature (60 °C). The percent RSD was found to be less than 0.7%, with no variation at lower temperature. However, 5% variation was observed at higher temperature and +0.1 min difference in retention time. The percent recovery was found to be within acceptable limits (95%–105%).

3.1.2.8. Mobile phase composition. Experiments were conducted using system suitability samples with mobile phase composition variation of +20% from the set percentage of TFA (0.1%). The percent variation between unaltered/initial condition and altered condition for EPO sample (done in triplicate) was found to vary less than 2.0% and there was no variation in retention time.

3.2. UPLC method

3.2.1. Method development

The basic chromatographic conditions like stationary phase, solvents and UV detector, employed in HPLC were taken into account while developing the new UPLC method. The stationary phase C_{18} was chosen in order to have similar polarity as that used in HPLC method. The injection volume was scaled down by about 10 fold as used in HPLC. To get the optimum results, mobile phase flow rate was kept constant at 0.35 mL/min and column temperature was maintained at 60 °C.

The chromatographic separation was achieved as described in Section 2.3.

The applied chromatographic conditions permitted a good separation of HSA and EPO. Different concentrations of EPO in the range 2.5–150 µg with 2.5 mg/mL of HSA were studied and no interference of HSA and other excipients was observed during the analysis. Representative chromatograms are shown in Fig. 3.

The capacity factor (k') was 2.45 and 3.9 for the first and second peak respectively, while the resolution factor was 5.35. The asymmetry of the peak was found to be 5.63 and 1.57 for HSA and EPO respectively. Tailing factor was found to be 3.68 and 1.33 for HSA and EPO, respectively. The percent RSD of the main peak area for replicate injections of EPO standard was found to be below 2.0% while no variation in the retention time was observed (less than 0.1 min).

3.2.2. Method validation

3.2.2.1. Specificity. Separation selectivity is the ability of the method to elicit a response specific for the analyte in the presence of other components/substances that are present or are likely to be present with the analyte.

To address separation selectivity, 0.1 mg/mL of EPO in mobile phase (as positive control), HSA 2.5 mg/mL in dilution buffer, HSA 2.5 mg/mL in mobile phase, 0.1 mg/mL of EPO with 2.5 mg/mL HSA in dilution buffer, mobile phase (Blank), Milli Q water and dilution buffer were injected into UPLC column.

HSA in dilution buffer and mobile phase was considered as the matrix components. Interference by the matrix components was evaluated by spiking known amount of EPO IRS in dilution buffer with HSA. No interference of matrix components was observed.

3.2.2.2. Linearity and range. EPO IRS was used for preparation of different working concentrations ranging from 0.0025 to 0.150 mg/mL, each containing 2.5 mg/mL of HSA. The peak area was plotted as shown in Fig. 4. Calibration curves with concentration versus peak area were plotted with blank subtraction. The correlation coefficient, slopes and Y-intercepts and regression equation were determined and are shown in Table 1. The correlation coefficient was found to be 0.999. The percent RSD was found to be less than 2.0% while the percent recovery was found to be in the range of 97%–103%.

3.2.2.3. Accuracy. Accuracy (% recovery) was studied with different working concentrations ranging from 0.0025 to 0.150 mg/mL of EPO. Each solution in its formulation buffer was spiked with HSA at a concentration of 2.5 mg/mL. The percent recovery was found to be in the range of 96%–103%. The percent RSD was found to be less than 2.0%.

3.2.2.4. Precision. Precision was evaluated by inter-day (Repeatability) and intra-day (Intermediate precision) variation, and different makes of columns. Repeatability (five replicates) was assessed independently for each of the three different concentrations (0.02 mg/mL, 0.04 mg/mL and 0.1 mg/mL). The percent RSD between areas of all five

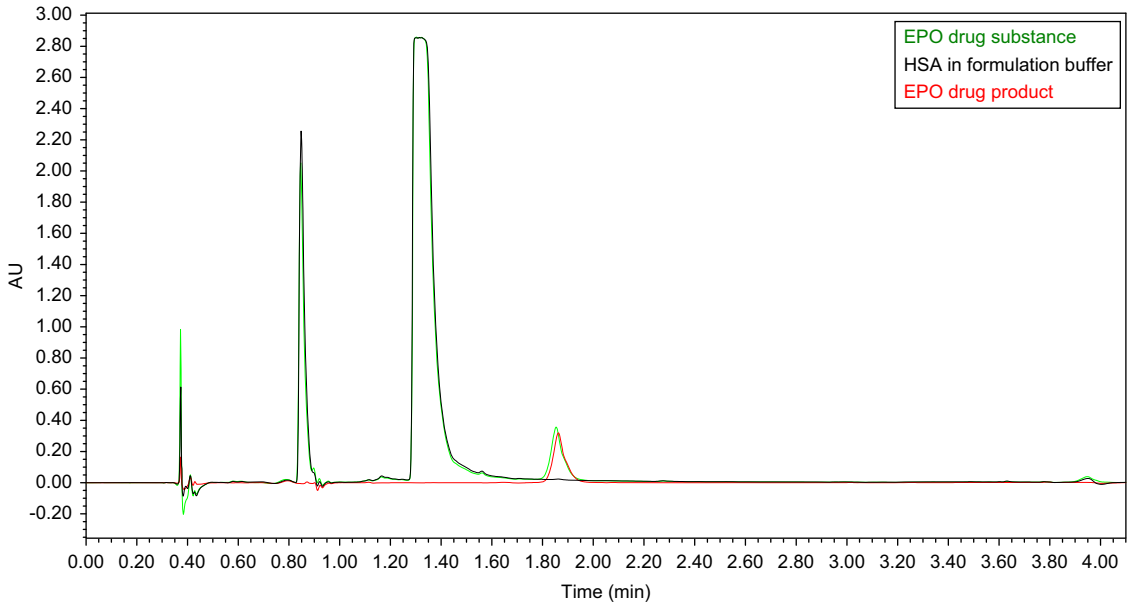

Figure 3 UPLC chromatogram of internal reference standard of EPO, HSA in formulation buffer (2.5 mg/mL) and EPO drug product.

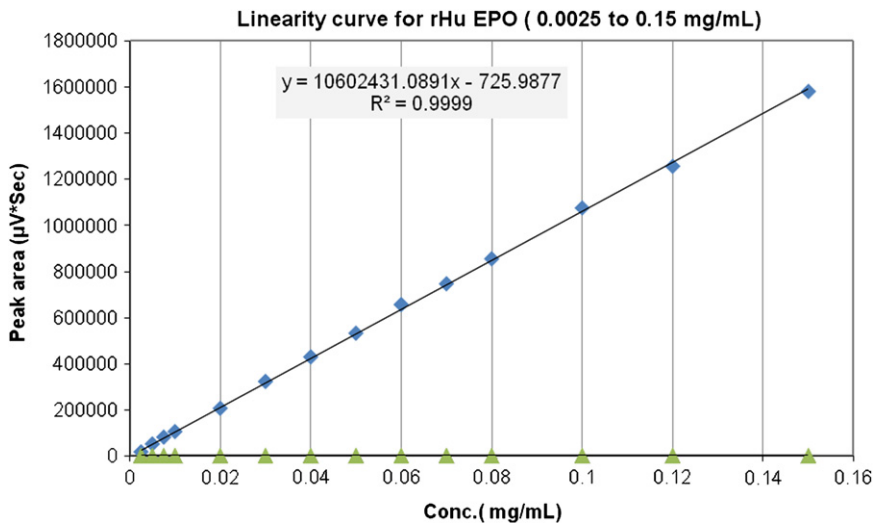

Figure 4 Linearity curve (UPLC) for EPO.

replicates was less than 1.5% for all dilutions and percent recovery of all five replicates was more than 95%.

3.2.2.5. Robustness. To determine the robustness of the method, experimental condition (TFA concentration) was purposefully altered and the resolution between EPO and HSA was examined. The TFA concentration was changed between 0.08% and 0.12%.

The percent recovery was found to be between 95% and 105%. The percent RSD for the area values obtained with altered and unaltered conditions of the parameter was found to be less than 2.5%, thus indicating that the developed method is robust and TFA concentration is not a critical parameter.

3.3. *Comparative study of HPLC and UPLC performance*

The performance parameters of both systems are shown in Table 1. The runtime of UPLC was reduced by 4-fold to that of HPLC. The retention behaviors of HSA and EPO were similar in HPLC and UPLC columns. As expected, the UPLC method showed higher efficiency of analysis than HPLC method.

4. Conclusion

Both RP-HPLC and RP-UPLC methods were demonstrated to be validated for quantifying EPO in presence of another protein (HSA), which is often present in medicinal formulations using HSA as stabilizer. The HPLC and UPLC methods were validated showing satisfactory data for all the parameters tested. The reported UPLC method was found to be capable of giving faster analysis with good resolution, accuracy and precision than that achieved with conventional HPLC method. Both the chromatographic methods were found to be reliable. Since these methods are rapid and simple, they may be

successfully applied to quality control analysis of EPO formulation containing HSA.

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