A HIGH-PERFORMANCE STABILITY-INDICATING LIQUID CHROMATOGRAPHIC NOVEL METHOD FOR DETERMINING RECOMBINANT HUMAN ERYTHROPOIETIN IN BULK AND DOSAGE FORM

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Abstract

An HPLC method for indicating stability was developed, created, and confirmed for the quantification measurement of recombinant human erythropoietin in large quantities as well as dosage forms. An isocratic separation was accomplished using a Thermo Biobasic C18 (250 mm 4.6 mm i.d., 300Ao, 5 m) column with a flow rate of 1.0 mL min^{-1} as well as a UV detector to monitor the eluate at 278 nm. Acetonitrile, 0.05 mM potassium dihydrogen phosphate (80:20 v/v), and orthophosphoric acid adjusted to 4.0 made up the mobile phase. The drug was exposed to oxidative stress, hydrolysis, and photodegradation to simulate stress conditionsRecombinant Human Erythropoietin, the parent compound, was eluted at roughly 6.675 minutes, as well as all byproducts have been entirely disconnected inside an overall analytical run time of about 15 minutes. To identify quantification limits of 0.05 and 0.2 g mL-1, respectively, the method was linear over a concentration range of 1-6 g mL-1 (r = 0.9989). Recombinant Human Erythropoietin can be measured accurately, selectively, sensitively, and precisely using this method both in dosage form and in bulk. Stress-induced degradation products did not interact with the identification of Recombinant Human Erythropoietin, indicating that the assay is stable.

Keywords: Recombinant Human Erythropoietin; Stability-indicating; Reverse phase; HPLC-UV.

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1. Introduction

Recombinant human Erythropoietin seems to be a 165 amino acid polypeptide chain nutritional hormone released in vivo by the kidney. The molecular weight of erythropoietin is approximately 34,000 Daltons. 60% of molecular weight is made up of protein. The compound contains four carbohydrate chains. These four carbohydrate chains are linked to the protein by three N-glycosidic bonds. There are four glycosylation sites in the amino acid sequence: one O-linked site at ser-126 and three N-linked locations at Asn-24, Asn-28, and Asn-83. The removal of sialic acid from oligosaccharides by enzymatic shortens EPO's biological half-life from 6-8 hours to a few minutes. [1] Erythropoietin (EPO) is indeed a glycoprotein hormone that is produced in the kidney and functions on bone marrow erythroid progenitor cells. Homeostasis in oxygen delivery to body tissues is provided by a negative feedback system wherein tissue oxygenation tries to control Epo output and Epo helps to control red blood cell (RBC) production. Decided to commit erythroid progenitor cells with specific Epo receptors are the target cells for the hormone's action. The EPO receptor is a member of the hematopoietic growth factor receptor family. There is no known second messenger system involved in signal transduction from the Epo receptor. Although Epo may have an impact on cell division in initial erythroid progenitor cells, its regulation of RBC production appears to take place later in the development of erythroid cells. [2] Subcutaneous injection results in lower serum erythropoietin concentrations than intravenous injection. The compound's levels in the serum gradually rise, reaching a peak 12 to 18-hour shifts after the dose. The half-life after subcutaneous injection is approximately 24 hours. Using a DNA Pac PA-100 analytical column, a high-performance anion-exchange chromatography (HPAEC) method to determine recombinant human erythropoietin (EPO) in pharmaceutical products was developed. [3] Over 100 minutes, another phase of HPLC is performed on a c-4 Vydac column (250.455cm) with an eluent consisting of a 0 to 95% acetonitrile gradient in 0.01 to

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1% trifluoroacetic acid. [4] The protein and carbohydrate substituents of EPO are modified by oxidation, which reduces its bioactivity. When EPO was treated with oxygen radicals produced by a Fenton system in the presence of 0.016mm H2O2, in vitro bioactivity was reduced, and the lowering has been directly related to the loss of in vivo bioactivity. [5] To the best of our knowledge, no analytical method for determining the stability of recombinant human erythropoietin in bulk or dosage forms has been published. As a result, a simple, precise, accurate, specific stability-indicating HPLC-UV method for quantification of Recombinant Human Erythropoietin in pharmaceutical forms has been invented and applied to the assay of Recombinant Human Erythropoietin in bulk and dosage form was developed and applied.

2. Materials and methods

2.1 Chemicals and Materials

Recombinant human Erythropoietin and its formulation Wepox were kindly given as gift samples by the Wockhardt research center, Aurangabad, India. Wepox injection was used as the reference formulation. Hcl, sodium hydroxide, hydrogen peroxide, orthophosphoric acid, and acetonitrile of HPLC grade were purchased from Merck. Every chemical was of analytical reagent grade and was used exactly as it was given. All solutions were made using reverse osmosis and filtration through a Milli-Q®system (Millipore, Milford, MA, USA) to obtain purified HPLC-grade water.

2.2 HPLC Instrumentation

The Jasco HPLC system consists of a pump with an injecting facility programmed for a 20 l capacity per injection (model Jasco PU2080, intelligent HPLC pump). The detector uses a UV/VIS (Jasco UV 2075) design that operates at a 267 nm wavelength. Jasco Borwin version 1.5, LC-Net II/ADC system, was the program used. Thermo Biobasic C18 columns (250 mm 4.6 mm i.d., 300Ao, 5 m) were used. A mobile phase made of acetonitrile, 0.05 mM

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dipotassium hydrogen phosphate (80:20 v/v), pH modified to 4.0 with orthophosphoric acid, and a flow rate of 1.0 mL min-1 was used to achieve the separation. UV identification at a wavenumber of 278 nm was used to keep an eye on the effluent. Before use, the mobile phase has been filtered through a 0.45 m nylon filter. In Table 1, HPLC conditions are listed.

2.3 Preparation of Stock and Standard solutions

By precisely measuring 25 mg of a true standard of recombinant human erythropoietin in a 25 mL A-grade volumetric flask and adding HPLC grade acetonitrile to volume, a stock solution of recombinant human erythropoietin (1000 g mL^{-1}) was created. Aluminum foil was used to block light from reaching the stock solution. A-grade bulb pipettes were used to transfer serial dilutions of the stock solution of recombinant human erythropoietin into 10 mL volumetric flasks, and the solutions were then diluted to quantity with the solvent system to give final concentrations of 1, 2, 3, 4, and 5 g mL^{-1}.

2.4 Forced degradation studies

Recombinant Human Erythropoietin pharmaceutical active ingredient powder was asserted under various conditions to conduct forced degradation studies to ascertain if the analytical method and assay were stability indicating [6]. The beginning concentration levels of recombinant human erythropoietin used in all solutions created for forced degradation studies were 1000 ng mL^{-1}.

2.4.1 Oxidation

To facilitate the oxidative stress of the Recombinant Human Erythropoietin, solutions for oxidation studies were made in acetonitrile and 6% H2O2 (20:80, v/v), and the resulting solutions were left at room temperature for 12 hours.

2.4.2. Acid degradation studies

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Acetonitrile and 2 N HCL (20:80, v/v) solutions were created for acid degradation studies, and the finished product was left to sit at room temperature for 24 hours.

2.4.3. Alkali degradation studies

Acetonitrile and 0.1 N sodium hydroxide (20:80, v/v) were combined to create solutions for alkali degradation studies, which were then left to sit at room temperature for 12 hours.

2.4.4. Freeze Thaw induced degradation studies

Alternatives to Freeze Studies on thaw-induced degradation were conducted using acetonitrile (20:80, v/v) as the preparation medium. The resulting solutions were then subjected to three cycles of freezing (-40^{0}C) and thawing (40^{0}C), lasting 24 hours each.

2.5.6. Photostability

To ascertain the impacts of UV light as well as sunlight ionizing radiation on the stability of recombinant human erythropoietin in solution and the solid state, recombinant human erythropoietin particles and solution were prepared and exposed to UV light (7300lux) and sunlight. A layer of less than 2 mm thick Recombinant Human Erythropoietin particles containing about 25 mg was applied to a glass dish. Recombinant Human Erythropoietin was dissolved in acetonitrile at a concentration of 1000 µg/mL. All samples were exposed to UV light and sunlight for 12 days while being tested for photostability. Additionally, control samples that were covered in aluminum foil were simultaneously exposed to UV and sunlight. All samples were cleaned of UV and sunlight before being ready for analysis as previously mentioned.

3. Results and discussion

3.1 HPLC method development and optimization

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The stressed samples were first put through an analysis with a mobile phase made of acetonitrile and trifluoroacetic acid pH 4.0 in a volume fraction of (70:30, v/v) at a constant flow rate mL min^{-1}. Since the separation and peak shape were not ideal under these circumstances, 0.05 mM potassium dihydrogen phosphate pH modified to 4.0 with orthophosphoric acid in the ratio of (80:20, v/v) was used in place of trifluoroacetic acid. With proper peak asymmetry and full baseline resolution, the overall chromatography improved as a result of changes in buffer and mobile phase proportion. The best chromatographic response was eventually found in a mobile phase of acetonitrile: 0.05 mM potassium dihydrogen phosphate (80:20, v/v) with pH adjusted to 4.0. This mobile phase was used for further research.

3.2 Validation

Several parameters, which include linearity, limits of quantitation (LOQ), limits of detection (LOD), precision, robustness, selectivity, and recovery, were considered in the method's validation [7-9].

3.2.1 Linearity

The Recombinant Human Erythropoietin calibration curves (n = 6) have been linear over the range of concentrations of 1-6 µg mL^{-1} as well as scanned at the wavenumber of 278 nm. As shown in Table 2, the peak areas of recombinant human erythropoietin have been obtained by plotting recombinant human erythropoietin concentration. The resulting curves were then subjected to linear regression analysis.

3.2.2 LOQ and LOD

The signal-to-noise ratios used to calculate the LOQ and LOD have been predicated on

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analytical responses that were ten and three times this same background noise, respectively. The LOD was discovered to be 0.05 µg mL^{-1} and the LOQ was determined to be 0.2 µg mL^{-1}.

3.2.3 Precision

Recombinant Human Erythropoietin was used to gauge the method's accuracy. Nine replicate applications and nine measurements of a stock solution at 3 distinct concentration levels 2, 4, and 6 µg mL^{-1} were used to assess the repeatability of the system. As shown in Table 3, the repeatability and intermediate precision studies' RSD values for the developed method were 1.404% and 0.957%, respectively.

3.2.4 Selectivity

The outcomes of stress testing studies showed that this method has a high level of selectivity for recombinant human erythropoietin. Figures 2, 3, 4, 5, 6, and 7 display typical chromatograms that were produced just after the test of pure bulk samples and stressed samples.

3.2.5 Robustness

An analytical procedure's robustness, which measures its ability to be unaffected by minor but intentional changes in method parameters, gives a clue as to how reliable it will be under typical conditions. Here, three variables that are mentioned throughout Table No. 5 were slightly altered. The 2, 4, and 6 µg mL^{-1} solution was used in this study.

3.2.7 Stability studies

Recombinant human erythropoietin was discovered to be stable under thermal (dry and wet heat) and neutral conditions, but it degrades when exposed to acid, base, photostability,

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freeze-thaw cycle, and oxidation conditions. By making three different amounts of Recombinant Human Erythropoietin 2, 4, and 6 µg mL^{-1} and storing them at room temperature for a day, it is possible to assess the stability of both the standard and sample solutions. After being injected into the HPLC system, there was no further peak visible in the chromatogram or quantification of recombinant human erythropoietin, indicating that it remained stable in the standard and sample solutions for up to three days at room temperature.

4. Conclusion

Recombinant Human Erythropoietin has been identified using a valid and reliable stability-indicating HPLC analytic approach in both bulk drug and dosage form. The method is selective and stability-indicating, according to the results of stress testing carried out by the International Conference on Harmonization (ICH) guidelines. The suggested technique is easy to use, accurate, precise, and capable of separating the drug from excipients and degradation products that are present in dosage forms. The technique can be used to analyze recombinant human erythropoietin in bulk powder or pharmaceutical dosage form regularly. The method's simplicity makes it suitable for use in labs without sophisticated analytical equipment like LC-MS or GC-MS, or where more complicated, expensive, and time-consuming methods are used. To forecast the expiration dates of pharmaceuticals, the HPLC procedure can also be used to analyze samples extracted during accelerated stability experiments.

5. Acknowledgement

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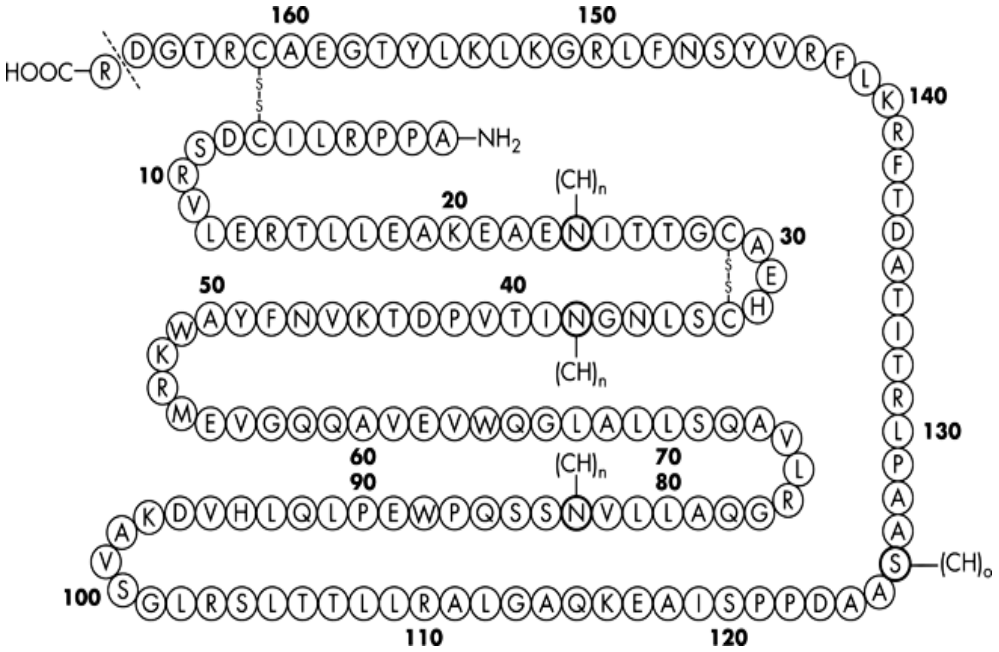

Fig. 1: Structural formula of Recombinant Human Erythropoietin (MW= 34,000 Daltons)

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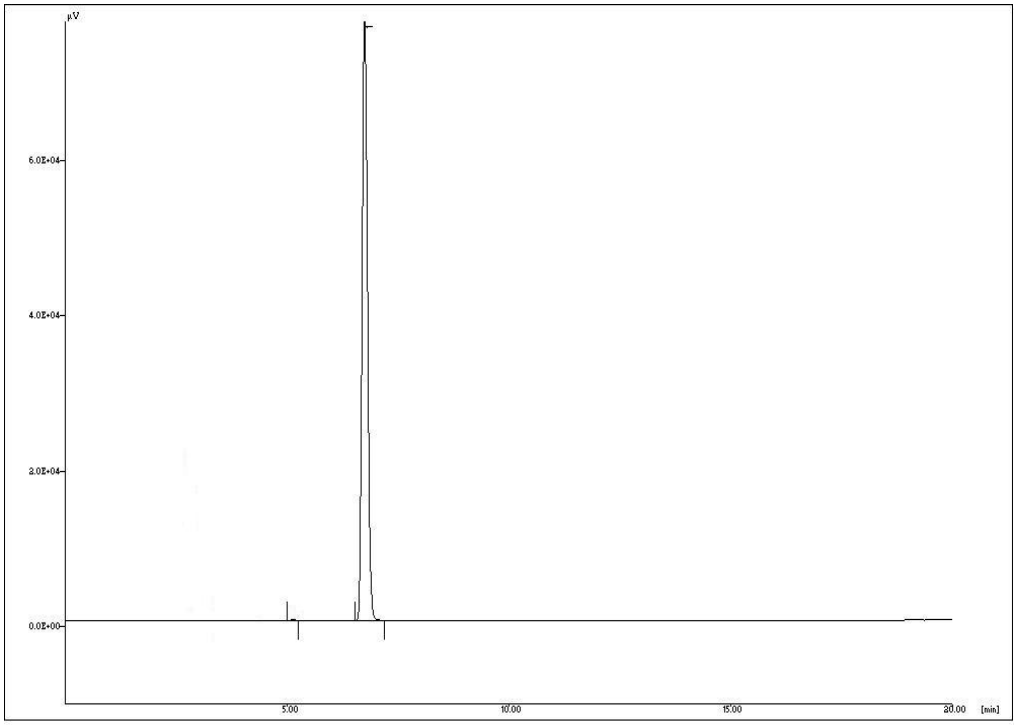

Fig.2: A RP-HPLC Chromatogram of a standard drug (10 µg mL^{-1}) ;(t_R :6.673)

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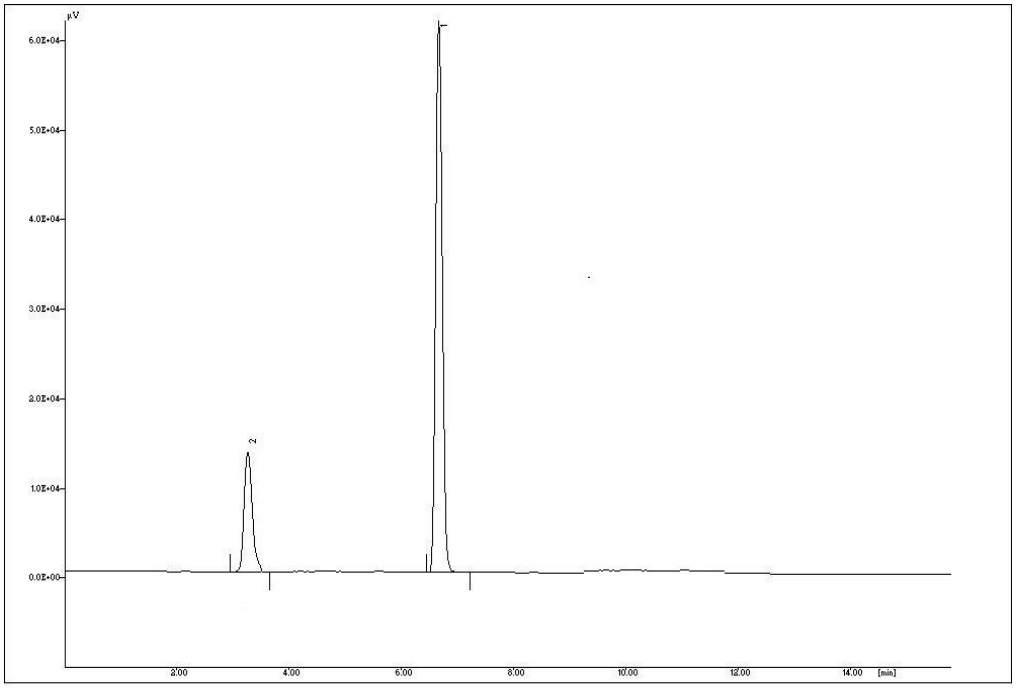

Fig. 3: Chromatogram of Recombinant Human Erythropoietin (10 µg/ml) under acidic stress condition, Degradant t_R = 3.42 min, erythropoietin t_R = 6.67 min

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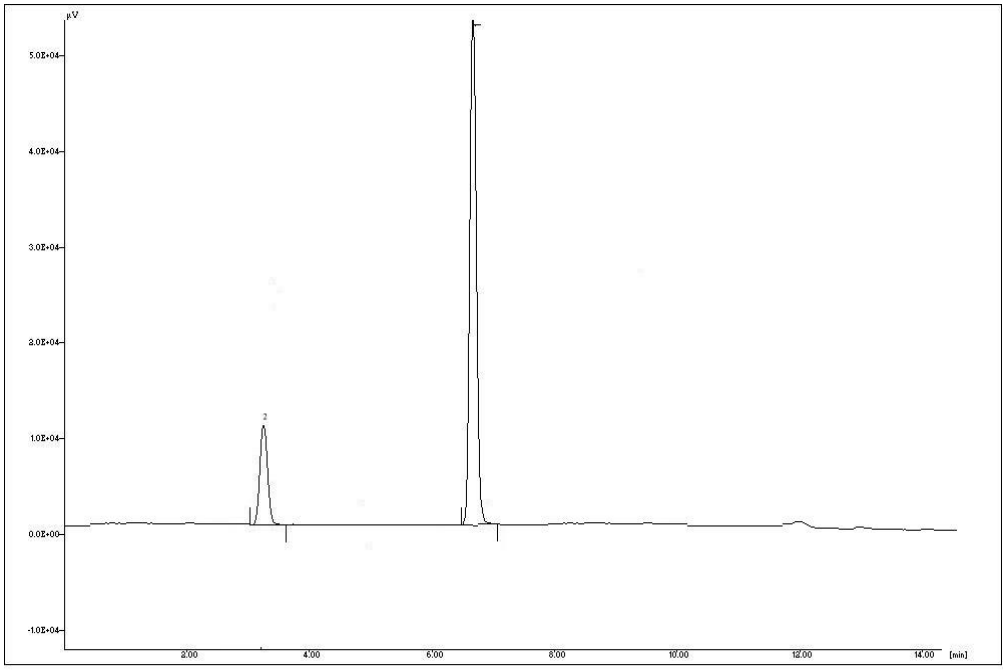

Fig. 4: Chromatogram of Recombinant Human Erythropoietin (10 µg/ml) under basic stress condition, Degradant t_R = 3.73 min, erythropoietin t_R = 6.69 min

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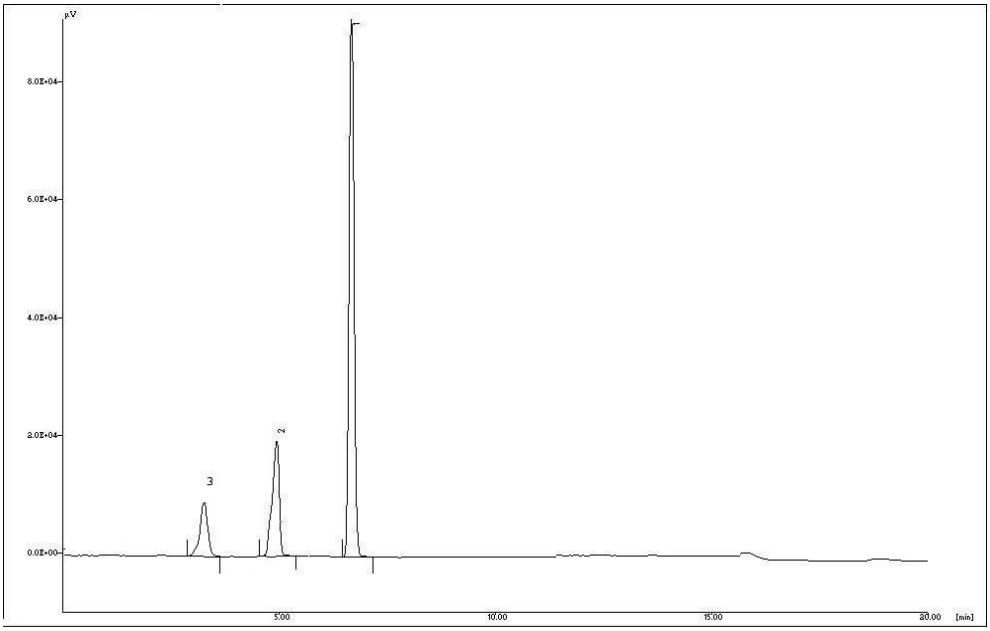

Fig. 5: Chromatogram of Recombinant Human Erythropoietin (10 µg/ml) under oxidative stress conditions,

Degradant-1 (OXID-1) t_R = 3.23 min, Degradant-2(OXID-2) t_R = 4.96 min and erythropoietin t_R = 6.62 min

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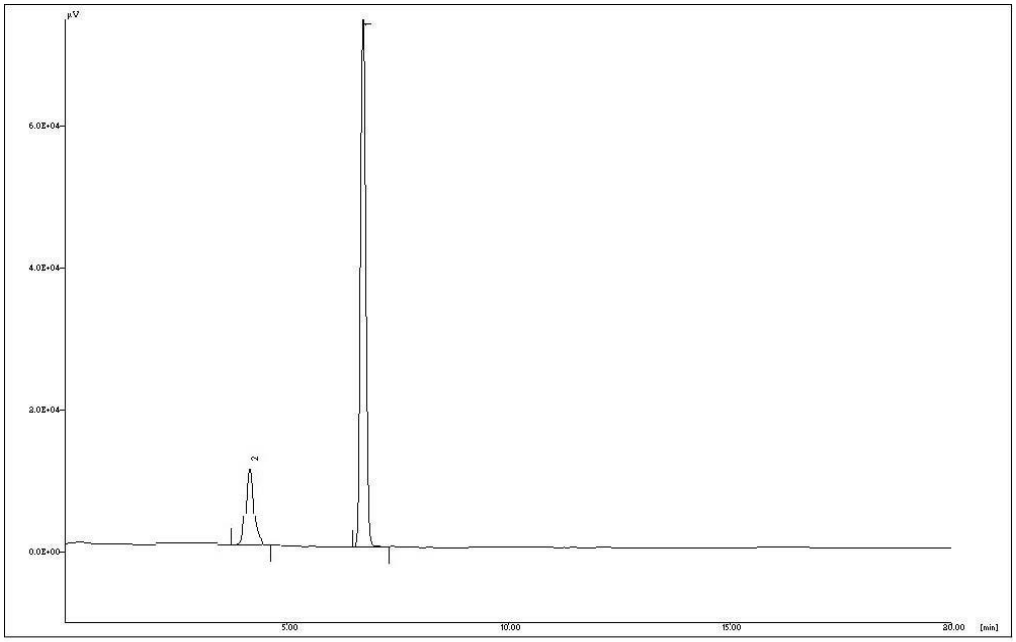

Fig. 6: Chromatogram of Recombinant Human Erythropoietin (10 µg/ml) under photodegradation stress condition, Degradant t_R = 4.68 min, erythropoietin t_R = 6.69 min

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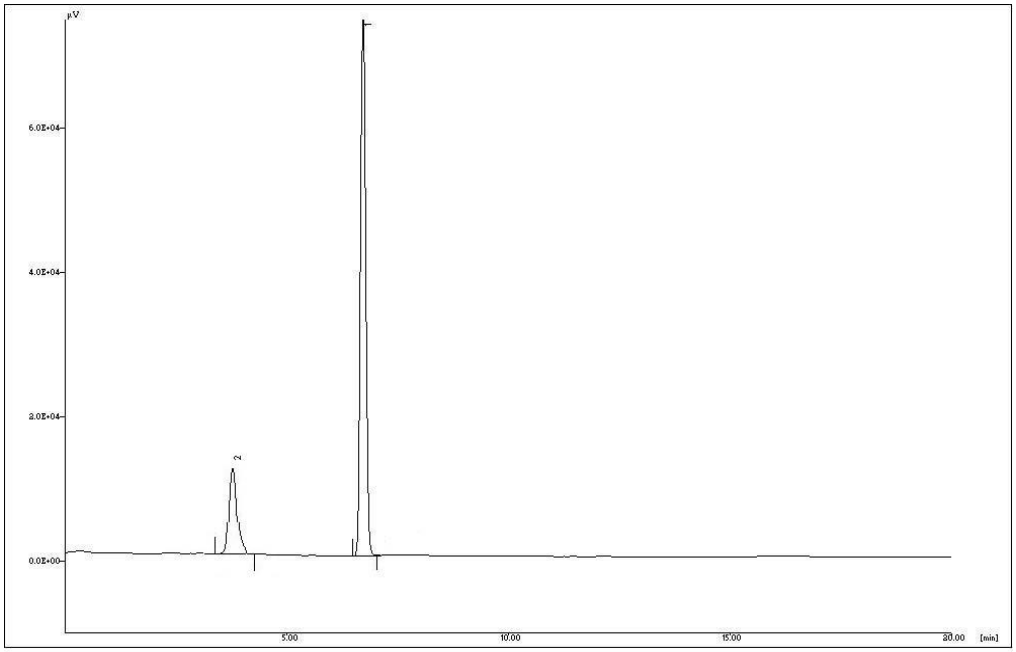

Fig. 7: Chromatogram of Recombinant Human Erythropoietin (10 µg/ml) under freeze-thaw induced stress condition, Degradant t_R = 4.32 min, erythropoietin t_R = 6.67 min

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Table 1: HPLC Conditions

Parameters	HPLC Conditions
Column	Thermo Hypersil Gold C18 (250 × 4.6 mm i.d., 300A^{o} 5µm)
Detector	278 nm
Injection Volume	20 µL
Flow Rate	1.0 mL min^{-1}
Temperature	25°C
Run Time	20 min
Mobile Phase	Acetonitrile: 0.05mM potassium dihydrogen phosphate (80:20v/v) pH adjusted to 4.0 with orthophosphoric acid.

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Table 2: Linear regression data for the calibration curve (n=6)

Parameter	HPLC
Linearity range	1-6 µg mL^1
r^2	0.9989
Slope	94368
Intercept	101447

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Table 3: Intra- and inter-day precision (n=9)

Concentration (µg/ml)	Repeatability (*n* = 9)		Intermediate precision (*n* = 9)	
	Found Concentration (µg/ml) ± S.D	RSD (%)	Found Concentration (µg/ml) ± S.D	RSD (%)
2	2.00 ± 0.026	1.326	1.99 ± 0.022	1.117
4	3.98 ± 0.075	1.890	4.02 ± 0.058	1.466
6	5.96 ± 0.059	0.996	5.98 ± 0.017	0.290

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Table 4: Robustness evaluation of the HPLC method (n = 9)

FACTOR	LEVEL	CHROMATOGRAPHIC CHANGES	
		Rt	Tf
A: Flow rate (ml/min)			
0.9	-1	6.69	1.15
1	0	6.67	1.13
1.1	1	6.23	1.06
Mean ± SD(n=6)		6.53 ± 0.26	1.11 ±0.047
B: % of acetonitrile in the mobile phase(v/v)			
19	-1	6.68	1.21
20	0	6.67	1.10
21	1	6.66	1.13
Mean ± SD(n=6)		6.67 ± 0.01	1.14 ± 0.056
C: pH of Mobile Phase			
4.1	-1	6.68	1.10
4.0	0	6.67	1.12
3.9	1	6.65	1.18
Mean ± SD(n=6)		6.66± 0.015	1.13 ± 0.041
D: Solvents of different lots			
First lot		6.67	1.10
Second lot		5.80	1.15
Mean ± SD(n=6)		6.23 ± 0.615	1.12 ± 0.035

Rt =Retention time in min, **Tf**= Tailing factor.