

Analytical Method Development and Validation of Etanercept by UV and RP-UFLC Methods

Husna Kanwal Qureshi, Ciddi Veeresham*, Chinta Srinivas

Department of Pharmaceutical Analysis, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India

Email: *ciddiveeresham@yahoo.co.in

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Abstract

The research work was carried out using Ultraviolet (UV)—visible spectroscopy and Reverse Phase-Ultra Fast Liquid Chromatography (RP-UFLC) for establishing novel methods for the analysis and quantification of Biosimilar drug, Etanercept. The maximum absorbance of Etanercept was found to be 215 nm and it obeyed Beer-Lamberts law in the range of 5 to 200 µg/ml and 1 to 32 µg/ml for UV and RP-UFLC, respectively. The correlation coefficient (r^2) value was found to be between 0.999 and 0.9995. All the validation parameters like linearity, accuracy, and precision, Limit of Detection (LOD), Limit of Quantitation (LOQ) and Robustness were found to be within acceptance criteria as per ICH guidelines. The results of accuracy studies (99.0% to 100.38%) indicated the methods to be accurate. The RSD % for interday and intraday precision studies was found to be less than 2%. Robustness and ruggedness were expressed in terms of RSD % which were also in the specified limits. LOD and LOQ of proposed method was calculated and found to be 1.257 and 3.809 µg/ml by UV, and 0.1073 µg/ml and 0.3251 µg/ml by RP-UFLC method, respectively. The developed methods were observed to be simple, rapid and cost-efficient. It can be easily applied for the estimation of Etanercept in the marketed formulations and for routine analysis of the Biosimilar drug.

Keywords

Biosimilars, Etanercept, Etacept[®], RP-UFLC, UV Spectroscopy, Validation

1. Introduction

Biosimilars are similar but not identical versions of a commercial originator/ innovator biotherapeutic that is being produced by different pharmaceutical manufacturers after patent and exclusivity expiration [1]. As per European Medicine Agency (EMA), Biosimilar drugs should exhibit similarity in terms of structure,

post-translational modifications, glycosylation and biological indications. Examples of Biosimilars of Etanercept like SB4, GP2015, HD203, LBEC0101, YLB113, etc.

The dawn of biological drugs has renewed the management and treatment of immune-mediated inflammatory diseases [2] [3] [4] [5] [6]. In order to target the pathogenic process of autoimmune rheumatic diseases, a class of biological Disease-Modifying Antirheumatic Drugs (DMARDs) has been introduced [3]. Examples include Tumor Necrosis Factor (TNF) inhibitors like Etanercept, Infliximab, Adalimumab, Golimumab and Certolizumab Pegol [7] [8]. It has revolutionized the treatment of rheumatic diseases [9] [10] [11].

Etanercept is a dimeric fusion protein-drug produced by recombinant DNA technology from the Chinese Hamster Ovary expression system [8] [12] [13] (Figure 1). It consists of 934 amino acids with a molecular weight of 150 kDa [14]. Its function is similar to the activity of naturally occurring soluble TNF alpha receptors [12]. It competes with TNF alpha cell membrane receptors preventing them from interacting with the pro-inflammatory cytokine. Thus, it decreases the local and systemic production of pro-inflammatory cytokines and subsequent effects [15]. It is used for the following indications like psoriatic arthritis, axial spondyloarthritis, plaque psoriasis, juvenile idiopathic arthritis/poly articular course juvenile rheumatoid arthritis and ankylosing spondylitis.

Literature survey [16] [17] [18] [19] revealed the availability of various methods and techniques used for comparative studies between innovator drugs and new biosimilars.

The literature search also revealed that there are no publications available on method development and validation on Etanercept by UV and RP-HPLC, so far to the knowledge of authors. Ultraviolet (UV) spectroscopy has the precedence

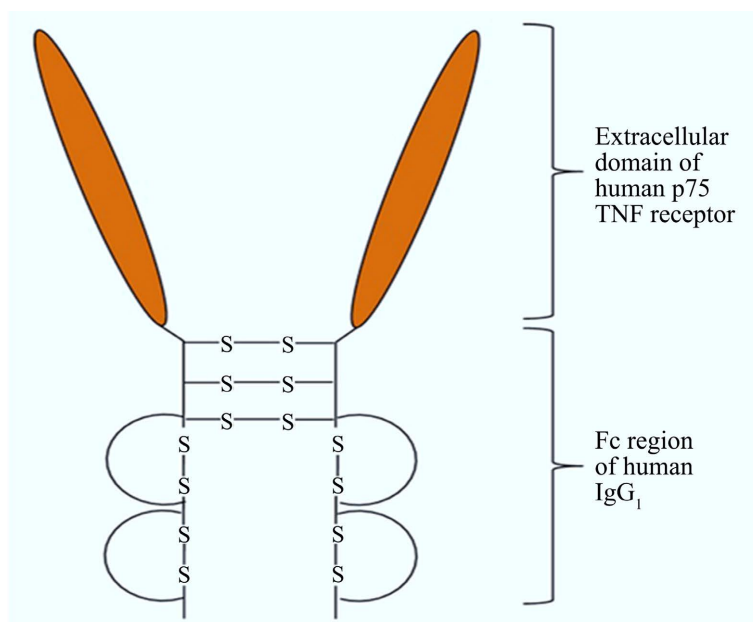


Figure 1. Structure of Etanercept.

of being simple, rapid and cost-effective. In view of this, the present paper aimed at the analytical method development along with validation of Etanercept by using UV spectroscopy and RP-UFLC.

2. Materials and Methods

2.1. Instrumentation

A Shimadzu UV1800 Spectrophotometer ENG 240V (Serial number: A11635101549) with Quartz cells of 1 cm path length and Shimadzu Prominence LC-20AD UFLC with UV detection (Model: RF-20A; Serial number: L20495001669) from Shimadzu Corporation (manufacturer), were used for the analysis. Lab solutions software for both instruments were used for analysis. Weighing balance (Shimadzu, Model: AUX 220) with internal automated calibration mode was used for the accurate weighing purpose.

2.2. Chemicals and Reagents

Etanercept reference standard was purchased from European Medicine Agency (EMA), Strasbourg, France. Etacept[®], marketed formulation was obtained as gift sample from Cipla Limited, Mumbai. Double distilled water was obtained from Merck Millipore Direct Q UV water system.

3. Methodology

3.1. Determination of Wavelength (λ_{\max})

The diluted standard solution (500 $\mu\text{g/ml}$) was scanned from 800 - 200 nm using UV/VIS Spectrophotometer, with double distilled water as blank. After acquiring the spectrum, λ_{\max} was identified. The above method was repeated thrice. The method was developed at room temperature (25°C).

3.2. RP-UFLC Chromatographic Conditions

The reverse-phase chromatographic analysis was done by using Phenomenex C18 column (25 cm \times 0.46 cm internal diameter) 5 μ , 100 Å analytical column with 0.1M Potassium dihydrogen Ortho phosphate buffer (pH-5.5) and Acetonitrile (60:40 v/v) as mobile phase in binary elution. The method was run at 1 ml/min at 215 nm.

3.3. Preparation of Working Standard Drug Solution

For preparing working standard solutions, Reference standard Etanercept (5 mg) was accurately weighed and transferred into the (5 ml) Volumetric Flask and dissolved properly to get 1 mg/ml stock solution. 5, 25, 50, 100, 150 and 200 $\mu\text{g/ml}$ were prepared from the standard stock solution for UV analysis and 1, 2, 4, 8, 16 and 32 $\mu\text{g/ml}$ were prepared for RP-UFLC method.

3.4. Preparation of Calibration Curve

The Calibration curve was developed by using 6 different dilutions prepared

from standard solution (5 to 200 µg/mL strength for UV and 1 to 32 µg/ml for RP-UFLC). Absorbance (UV) and peak areas (RP-UFLC) of every calibration standard were estimated at λ_{\max} of 215 nm. The calibration curves representing Absorbance/Peak areas vs concentrations were plotted using Microsoft Excel 2013.

4. Method Validation

Developed UV and RP-UFLC methods were validated as per specifications from International Conference on Harmonization (ICH) for analytical validation. The methods were analyzed for the parameters like linearity, accuracy, precision, robustness, ruggedness, LOD and LOQ.

4.1. System Suitability

For evaluating the suitability of UFLC system and developed procedure, Etanercept standard solution of 10 µg/ml concentration was prepared and about 20 µl was injected into the UFLC system. Then the chromatogram was recorded.

4.2. Linearity

For demonstrating linearity, six different concentrations were analyzed each for the optimized methods. 5, 25, 50, 100, 150 and 200 µg/ml were concentrations for UV and 1, 2, 4, 8, 16 and 32 µg/ml concentrations for RP-UFLC. After analyzing calibration standards, calibration curve in terms of absorbance vs. concentration was developed for UV method and Peak area vs. concentration for RP-UFLC. Both were subjected to linear least square regression analysis.

4.3. Accuracy

The accuracy was assessed by carrying out recovery studies (standard addition method) for the proposed UV and RP-UFLC methods. For this, three different concentrations of Etanercept were prepared in triplicate at level of 50%, 100% and 150% of its predefined concentration (50, 100, 150 µg/mL). Accuracy was determined based on percent recovery.

4.4. Precision

Precision expresses the reproducibility of the measurements and is determined in terms of repeatability. It is analyzed by carrying out six independent assays of Etanercept solution (intra-day). Intermediate precision was analyzed by repeating same method on following three days. Both Intraday and Interday precision are expressed in terms of RSD %.

4.5. Robustness

Robustness of analytical method is the capability of an optimized method to remain unchanged in its execution despite of slight, minimal changes in method parameters. Robustness of the developed UV method was obtained by changing

wavelength (± 1 nm) and observing the absorbance whereas for RP-UFLC method it was assessed by changing flow rate by ± 0.1 ml and slight variation in the optimized mobile phase ratio. The results were expressed in terms of RSD %.

4.6. Ruggedness

Ruggedness of analytical method is the ability of a method to perform unaffected in presence of various external influences. Rugged analytical methods are given priority as they are free from influence of surrounding environmental factors. The UV and RP-UFLC method was carried out by analyzing Etanercept samples in triplicate and by utilizing two distinct instruments. The results were calculated in terms of RSD %.

4.7. LOQ and LOD

For determining LOQ, following equation was used.

$$\text{LOQ} = 10 \times \text{SD/S}$$

For determining LOD, following equation was used.

$$\text{LOD} = 3.3 \times \text{SD/S}$$

where, SD = Standard deviation; S = Slope

4.8. Assay of Etanercept Content in Marketed Formulation

The proposed UV-Vis and RP-UFLC methods were promptly used for determination of Etanercept content in pharmaceutical marketed formulation. For the study, Etacept[®] 25 mg injection was used and suitable dilution was made using double distilled water. The prepared marketed formulation samples were analyzed using optimized and validated methods.

5. Results

5.1. Method Development and Optimization

Determination of maximum absorbance is the first step for the analysis (quantitatively or qualitatively) by UV and RP-UFLC. Etanercept solution (500 $\mu\text{g/mL}$) was scanned using UV-Visible spectrophotometer (**Figure 2**). UV software processed full scan and the λ_{max} were identified with the help of software to be 215 nm for Etanercept.

5.2. Preparation of Calibration Curve

For quantitative analysis of Etanercept, six concentrations namely 5, 25, 50, 100, 150 and 200 $\mu\text{g/ml}$ were used for developing calibration curve by UV. The absorbance of different calibration standards was measured at 215 nm using fixed wavelength mode of spectrophotometer. Absorbances of each concentration were analyzed in triplicate and for calibration curve the average of the absorbances were taken. Equation $y = 0.0021x + 0.0046$ was obtained for the absorbance plotted against different concentrations of the drug (**Figure 3**). By RP-UFLC, Equation

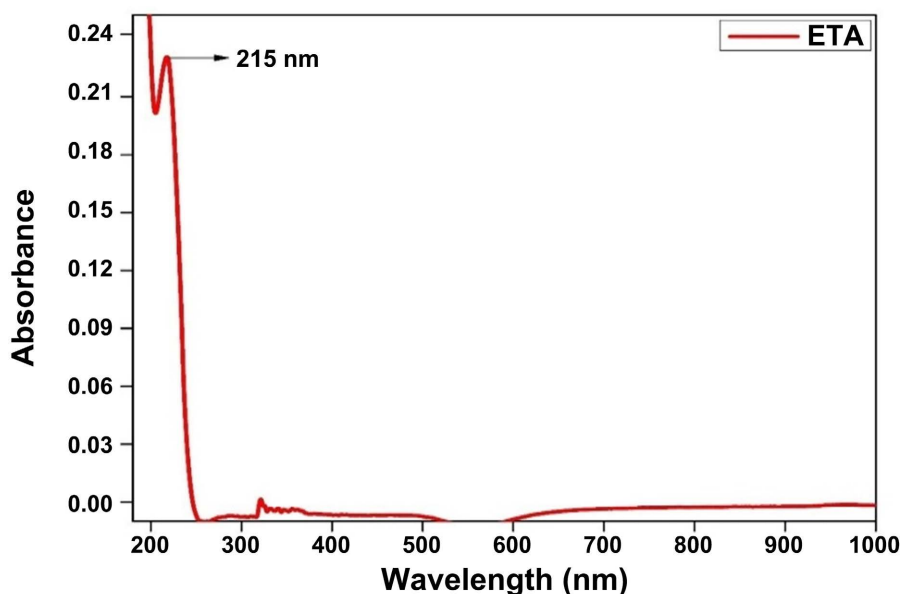


Figure 2. UV-visible spectra of Etanercept reference standard (ETA).

$y = 83,337x + 45,060$ was obtained for the peak areas plotted against different concentrations (1, 2, 4, 8, 16 and 32 $\mu\text{g/ml}$) (**Figure 4**).

5.3. Method Validation

5.3.1. System Suitability

As per the ICH guidelines, the theoretical plate number (greater than 2000 *i.e.*, 6837 ($n = 6$)), Tailing factor (<2 , *i.e.*, 1.309 ($n = 6$)) and percentage relative standard deviation ($\leq 2\%$) were obtained and demonstrated that the method can generate the accurate and precise results with optimized conditions (**Figure 5**).

5.3.2. Linearity and Range

For UV analysis, Calibration curve of Etanercept covering a range of 5 to 200 $\mu\text{g/ml}$ was developed (**Figure 3**). Correlation coefficient value was found to be 0.999 for the calibration curve which yielded an equation, $y = 0.0021x + 0.0046$ upon subjugation to least square regression analysis.

For RP-UFLC analysis, Calibration curve was in the range of 1 to 32 $\mu\text{g/ml}$, with Linear equation found to be $y = 83,337x + 45,060$. The regression coefficient was considered to be 0.9995 (**Figure 4**). Retention time was found to be 3.18 ± 0.01 min (**Figure 5**). The linearity graph, disclosed that the developed methods are linear in the mentioned concentration range of calibration standards.

5.3.3. Accuracy

For UV and RP-UFLC methods for Etanercept analysis, accuracy was assessed using recovery studies. Range of mean recovery of Etanercept was found to be in between 99.8% to 100.38% (UV) and 99% to 100.166% (RP-UFLC) (**Table 1**). From the accuracy studies, it was revealed that developed UV and RP-UFLC methods are accurate as the RSD % was below 2%.

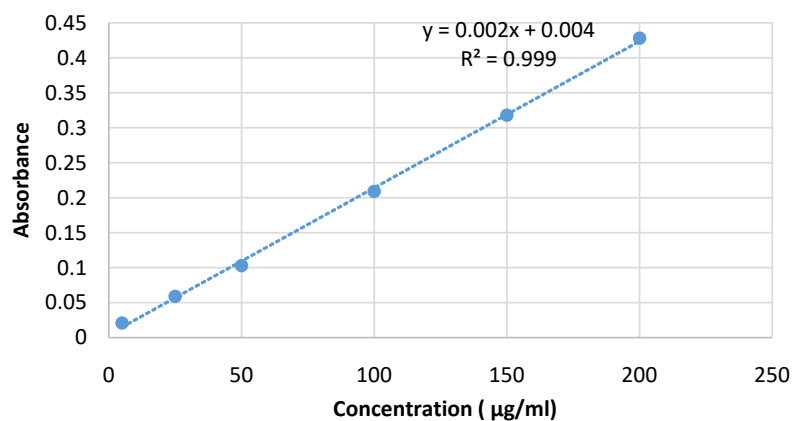


Figure 3. Calibration curve for Etanercept by UV spectroscopy.

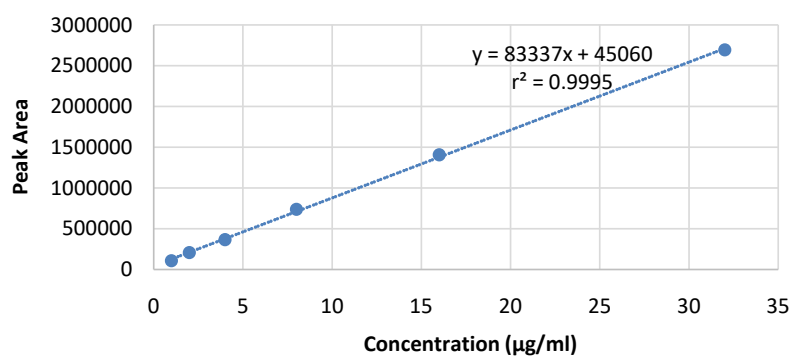


Figure 4. Calibration curve of Etanercept by RP-UFLC.

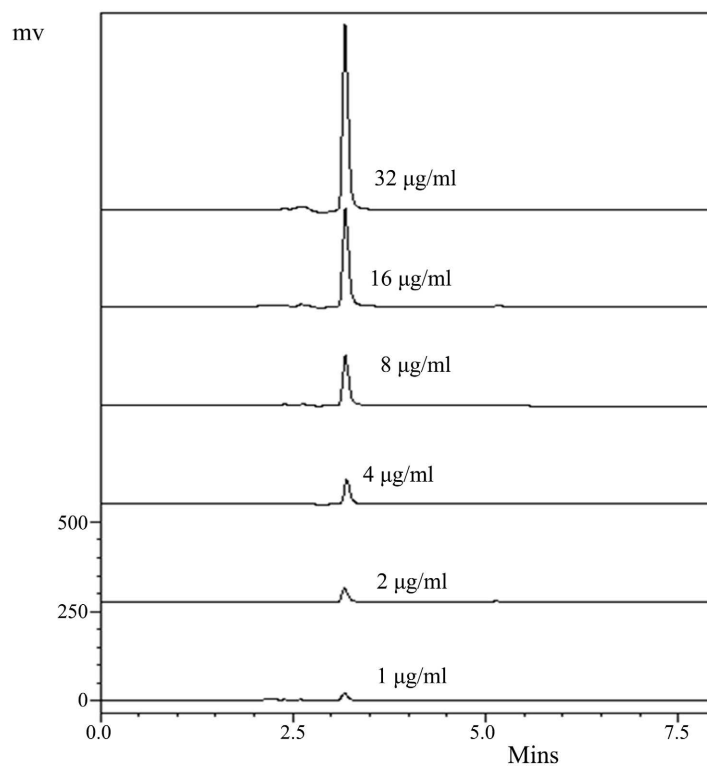


Figure 5. Chromatogram of Etanercept by RP-UFLC.

Table 1. Accuracy data of Etanercept by UV spectrometer and RP-UFLC.

S. No.	Conc. (%)	UV Spectrometry			RP-UFLC		
		% Recovery	Mean % Recovery	RSD %	Recovery %	Mean % Recovery	RSD %
1	50	100.38			99.15		
2	50	101.34	100.38	1	98.4	99	0.43
3	50	99.42			99.45		
4	100	100.1			99.375		
5	100	100.6	100.13	0.579	100.425	99.425	0.86
6	100	99.71			98.475		
7	150	99.49			718,631		
8	150	99.8	99.8	0.581	710,353	100.166	0.69
9	150	100.12			709,879		

5.3.4. Precision

Intra- and inter-day precision of developed methods were estimated at 25, 50 and 100 µg/ml levels of Etanercept for UV and 8, 16, 32 µg/ml for RP-UFLC. The results are presented in **Table 2** and **Table 3** and are explained by means of average/mean absorbance values, assay % and RSD % for the intra- and inter-day precision of both the methods. Inclusively, RSD % values were found to be lower than 2 which showed that the results are as per the guidelines.

5.3.5. Robustness

Robustness of the proposed methods was established by analyzing at different temperatures, with slight variation in flow rates and mobile phase ratios. RSD % values were found to be below 2 and in between 0.14 and 1.36 as shown in **Table 4** and **Table 5**.

5.3.6. Ruggedness

Ruggedness was assessed by analyzing Etanercept solution in two different UV spectrophotometers (**Table 6**) and two different HPLC systems (**Table 7**). RSD % values were between 0.27 and 0.84. RSD % was within the acceptable limits and also proposed that the methods were rugged.

5.3.7. LOQ and LOD

LOD and LOQ of proposed UV method was found to be 1.257 and 3.809 µg/ml, respectively whereas LOD and LOQ of RP-UFLC method was found to be 0.1073 µg/ml and 0.3251 µg/ml, respectively. Lesser LOQ value indicated that optimized method can be suitably used for analyzing the samples containing even minimal quantities of Etanercept.

5.3.8. Assay of Etanercept in Marketed Formulation

The developed UV and RP-UFLC method were applied for the estimation of Etanercept content in Etacept[®] 25 mg injection. Average percent assay of Etanercept

Table 2. Precision data of Etanercept by UV spectroscopy.

S. No.	Conc. (µg/ml)	Intraday Precision								
		Morning			Afternoon			Evening		
		Mean	Assay %	RSD %	Mean	Assay %	RSD %	Mean	Assay %	RSD %
1	25	0.058	101.71	1.72	0.0573	100.38	1.01	0.0567	99.23	1.02
2	50	0.109	99.42	0.92	0.110	100.38	0.91	0.1084	98.85	0.54
3	100	0.215	100.19	0.47	0.2143	99.85	0.27	0.2137	99.57	0.27
Interday Precision										
S. No.	Conc. (µg/ml)	Day 1			Day 2			Day 3		
		Mean	Assay %	RSD %	Mean	Assay %	RSD %	Mean	Assay %	RSD %
		1	25	0.0570	99.80	1.75	0.0570	99.80	1.75	0.0564
2	50	0.1094	99.80	0.53	0.1090	99.42	0.92	0.1077	98.47	0.54
3	100	0.2154	100.38	0.27	0.2147	100.04	0.27	0.2144	99.90	0.27

Table 3. Precision data of Etanercept by RP-UFLC.

S. No.	Conc. (µg/ml)	Intraday Precision								
		Morning			Afternoon			Evening		
		Mean	Assay %	RSD %	Mean	Assay %	RSD %	Mean	Assay %	RSD %
1	8	712,954.34	100.125	0.69	717218	100.75	0.23	716,060.67	100.625	0.09
2	16	1,402,589.67	101.75	0.09	1,403,482.33	101.875	0.04	1,402,693	101.812	0.32
3	32	2,694,083.33	99.331	0.07	2,694,278.33	99.312	0.07	2,694,393	99.343	0.15
Interday Precision										
S. No.	Conc. (µg/ml)	Day 1			Day 2			Day 3		
		Mean	Assay %	RSD %	Mean	Assay %	RSD %	Mean	Assay %	RSD %
		1	8	716,060.667	100.625	0.16	717,248.33	100.75	0.05	717,358.67
2	16	1,402,312.67	101.75	0.05	1,401,464.67	101.687	0.01	1,401,276.33	101.687	0.06
3	32	2,696,056	99.406	0.1	2,695,367	99.375	0.04	2,695,505.33	99.384	0.12

Table 4. Robustness data at different temperatures by UV spectroscopy.

S. No.	Conc. (µg/ml)	Temperature	Absorbance	% RSD
1	200	25°C	0.4277	0.14
2	200	28°C	0.4276	0.27

Table 5. Robustness data of Etanercept by RP-UFLC.

S. No.	Conc. (µg/ml)	Flow Rate (ml/min)	Mobile Phase Ratio (Buffer:ACN)	Rt	Peak Area (Mean)	% RSD
1	4	1	61:39	3.18	370,778	1.36
2	4	1	59:41	3.17	366,232.34	1.01
3	4	0.9	60:40	3.18	3,666,373	0.72
4	4	1.1	60:40	3.19	356,159	0.95

Table 6. Ruggedness data of UV method for Etanercept.

S. No.	Conc. ($\mu\text{g/ml}$)	Instruments Used	Absorbance	% RSD
1	100	UV 1800 Shimadzu spectrophotometer	0.2147	0.27
2	100	Elico Double beam SL 210 UV VIS spectrophotometer	0.2144	0.27

Table 7. Ruggedness data of RP-UFLC method for Etanercept.

S. No.	Conc. ($\mu\text{g/ml}$)	Instruments Used	Rt	Peak Area (Mean)	% RSD
1	2	Shimadzu Prominence LC-20AD UFLC system	3.17	210,185	0.35
2	2	Shimadzu LC 20 AD UFLC, Diode array detector	3.18	209,210	0.84

in Etacept[®] injection as per UV and RP-UFLC methods were found to be 100.76% and 100.25%, respectively which is within the acceptable limits set by ICH.

6. Discussion

Analysis of biologics involves the use of sophisticated and advanced analytical methods especially for comparing the innovator with the newly approved biosimilars. Analyzing the glycans [20], measurement of free and unoxidized thiols using 5,5'-dithionitrobenzoic acid (DTNB) and DyLight Maleimide (DLM) as derivatizing agents [21], detection of instability in Etanercept during thermal stress testing [17], physicochemical and clinical comparability followed by a clinical study [16] [18] [22] [23] [24], absolute quantification of oxidation in monoclonal antibodies and Fc-fusion proteins by UV and MS detection [25], etc. are to name a few intricate techniques used for comparisons. In collation with advanced technologies, UV and RP-UFLC methods can be used for quantitative analysis. Ultraviolet (UV) methods have the benefit of being simple, easy, rapid and cost-effective. In comparison to earlier reported work, the present RP-UFLC work deals with reliable and economic analysis of Etanercept. The optimized RP-UFLC method is evidenced to be less time-consuming (Rt 3.18 ± 0.1 min with binary elution). Reported literature employed gradient time programmed elution whereas binary elution was used in the proposed method. Protein biologics from different manufacturers can be analyzed using the developed RP-UFLC method.

In conclusion, faster analysis time, increased sensitivity, and cost-effectiveness of these methods demonstrated that they are suitable for routine laboratory use. Simple, reliable, quick, economic, accurate, precise and sensitive methods were developed and validated as per ICH guideline.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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