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Analytical Method Development and Validation of Some Biosimilar Drugs by High Performance Thin Layer Chromatography

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Abstract

A simple and rapid HPTLC analytical method has been developed and validated for the determination of Etanercept and Filgrastim in pure form and in marketed formulation. Both the drugs were chromatographed on silica gel 60 F254s HPTLC plates, as stationary phase. The mobile phase optimized for Filgrastim and Etanercept was Water: n-butanol (7.5:2.5 v/v) and Isopropyl alcohol: water (6.5:4.5 v/v), respectively. The chromatogram obtained was scanned at 225 nm and 222 nm for filgrastim and etanercept which resulted in a retention factor of 0.45 ± 0.07 and 0.32 ± 0.03, respectively. The method was validated for parameters like linearity, accuracy, precision, specificity and robustness. Recovery studies were performed at three concentration levels, to demonstrate suitability, accuracy and precision of proposed method. Statistical analysis proved that the proposed method is accurate and reproducible with linearity in the range of 500 to 3000 ng/band for filgrastim and 200 to 1200 ng/band for etanercept. The limit of detection and limit of quantification for filgrastim was found to be 63.418 ng/band and 192.177 ng/band. For etanercept, LOD and LOQ were found to be 33.381 ng/band and 101.153 ng/band, respectively. The proposed method can be employed for the routine analysis of selected biosimilars.

Keywords

Biosimilars, Etanercept, Filgrastim, Method Development, Validation

1. Introduction

Etanercept is a dimeric fusion protein drug produced by recombinant DNA technology from Chinese Hamster Ovaries expression system [1] [2]. It acts as a Tumour Necrosis Factor (TNF) inhibitor. Etanercept blocks the interaction of

TNF with receptors on the cell surface, henceforth preventing TNF-mediated immune and inflammatory responses. It belongs to the class of biological disease modifying anti-rheumatic drugs (bDMARDS).

Filgrastim or Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is one in the family of hematopoietic growth factors which regulates the proliferation and differentiation of cells of neutrophil lineage. It is produced by recombinant DNA technology in genetically engineered *Escherichia coli* cells. It is used for reducing the risk of infection in cancer patients by improving the neutrophil count and in patients receiving chemotherapy or a bone marrow transplant [3] [4].

High Performance Thin Layer Chromatography (HPTLC) is an analytical technique that includes a widely standardized and systematized methodology. It is used for qualitative and quantitative analysis and also for validation of not only plant extracts but also a number of pharmaceutical formulations [5] [6]. HPTLC meets all quality requirements as par with any analytical techniques. HPTLC can reproduce data with the best resolution and with more accurate quantitative measurements [6] [7]. HPTLC has the advantage of being reliable in quantitation [8], simple in procedure [9], and sensitive analysis not only in micrograms but also in nanograms levels above all being cost effective [6]. The volume of solvent required is very less in comparison to HPLC. This minimizes the time and expenditure of analysis. It also diminishes the possibilities of pollution of the environment. Concurrent assay of many components in a composite formulation or extracts is also possible [8] [10] [11]. Hence, the present research aimed to develop and validate a simple, rapid and specific HPTLC method for the determination of Etanercept and Filgrastim in accordance with International Conference on Harmonization (ICH) guidelines.

2. Materials and Method

2.1. Reagents Used

Etanercept and Filgrastim were obtained from European Medicine agency, Strasbourg, France. Etacept® and Grafeel®, marketed formulation was obtained as gift sample from Cipla Limited, Mumbai and Dr. Reddy's Laboratories, Hyderabad, India, respectively. Merck Millipore Direct Q UV water system was used to obtain double distilled water for analysis. Isopropyl alcohol and n-Butanol was purchased from Merck.

2.2. Instrumentation

CAMAG® Linomat 5 Automatic TLC Sampler 4 (ATS 4) with TLC scanner 3, Switzerland. Silica gel 60 RP-18 F254s HPTLC plates were obtained from Merck.

3. Methodology

3.1. Preparation of Standard Stock Solution

Standard stock solution of Etanercept was prepared by dissolving 10 mg of drug

in 10 ml of water to get concentration of 1000 µg/ml. From the standard stock solution, working standard solution was prepared containing 100 µg/ml of Etanercept.

Working stock solution containing 250 µg/ml of Filgrastim was prepared from standard filgrastim solution. The stock solution was used for detection of wavelength for maximum absorbance.

3.2. Selection of Detection Wavelength

Working stock solution was scanned over the range of 200 - 400 nm and the spectrum was obtained. It was observed that Etanercept showed considerable absorbance at 222 nm (Figure 1) and filgrastim showed at 225 nm. Representative UV spectrum of Filgrastim is shown in Figure 2.

3.3. HPTLC Analysis

Solution of Etanercept (100 µg/ml) and Filgrastim (200 µg/ml) was prepared. 4 µl (400 ng/band for etanercept) and (1000 ng/band of filgratim) of solution was applied on pre-activated HPTLC plate with the help of Hamilton syringe (100 µl), using Linomat 5 sample applicator. After spotting the plate, it was placed in the saturated chamber and developed up to 80 mm distance. The plate was dried and was scanned over 90 mm distance at 222 nm for etanercept and 225 nm for

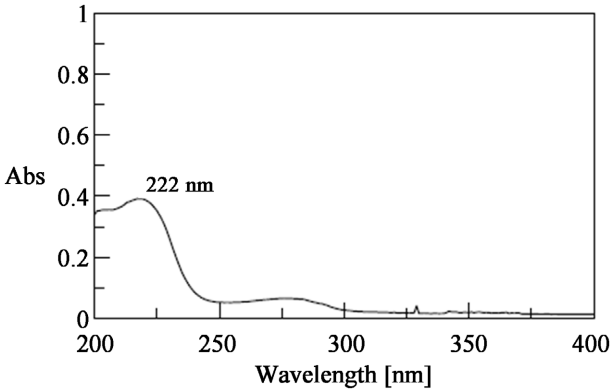


Figure 1. UV spectrum of Etanercept.

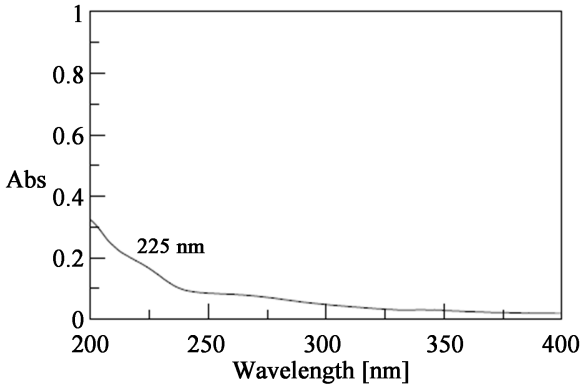


Figure 2. UV spectrum of Filgrastim.

filgrastim. The retention factor was found to be 0.32 ± 0.03 for Etanercept and 0.45 ± 0.07 for Filgrastim.

3.4. Validation

The HPTLC method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guideline [12] for linearity, precision, accuracy and recovery, limit of detection, limit of quantification, specificity and robustness.

3.4.1. Specificity

The specificity of the method was ascertained by analyzing standard compound and by peak purity profile studies

3.4.2. Linearity and Range

To evaluate linearity range, the calibration curve was plotted based on the peak area obtained against concentration of etanercept and Filgrastim. Scanning over the concentration range 200 - 1200 ng/ml for etanercept and 500 - 3000 ng/ml for Filgrastim was carried out. The correlation coefficient (r) values were calculated.

3.4.3. Precision

% RSD is used to estimate the Intraday and Interday precision of the method. Precision was calculated on the same day (Intraday) and on three consecutive days (Interday). The analysis was done in triplicate for each sample for both the drugs.

3.4.4. Accuracy

The standard addition method was used to determine the accuracy of the method. Known amounts of Etanercept and filgrastim was added at three different levels (50%, 100% and 150%) and analysis was carried out for both the drugs in triplicate.

3.4.5. Robustness

Robustness is a measure of the ability of a developed method to remain stable and unaffected by minor and deliberate changes in the experimental conditions. It indicates reliability of the method.

3.4.6. Limit of Detection (LOD) and Limit of Quantitation

They are calculated from the following formula,

$$\text{LOD} = 3.3\ \sigma / S$$

$$\text{LOQ} = 10\ \sigma / S$$

where, σ is the standard deviation of Y intercept and S is slope of the calibration curve.

3.4.7. Assay (Marketed Formulation)

Etacept® and Grafeel® pre filled injections were taken and a concentration equivalent to 400 ng/band for Etanercept and 1000 ng/ml for Filgrastim were analyzed from formulations, respectively. Concentration and % recovery were

determined. The sample was analyzed six times.

4. Results

4.1. HPTLC Analysis (Table 1)

The retention factor was found to be 0.32 ± 0.03 for ETA and 0.45 ± 0.07 for FILG.

4.2. Validation of Analytical Method

The proposed method was validated as per International Council for Harmonisation (ICH Q2 (R1)) guidelines.

4.2.1. Specificity

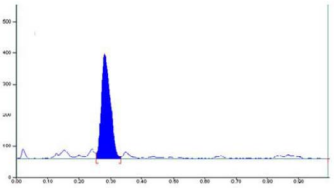
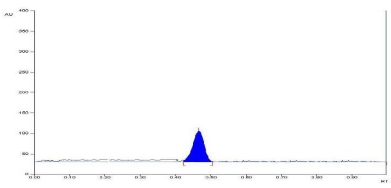
The specificity of the developed method was established by peak purity profiling studies. The peak purity values were found to be more than 0.996 for Etanercept and 0.998 for Filgrastim, indicating the non-interference of any other peak of degradation product or impurity.

4.2.2. Linearity

From the 100 µg/ml solution of Etanercept, five replicates per concentration were spotted. The linearity was determined by analyzing six concentrations over the concentration range of 200 - 1200 ng/band. Against the corresponding concentrations the peak areas were plotted to obtain the densitogram as shown in Figure 3. The result was found to be linear with regression equation of y = 9.0888x + 5321.3 (Figure 4) and r^2 value of 0.9905.

Working standard solution containing 250 µg/ml of Filgrastim was further used for preparing dilutions and spotting. Six replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was

Table 1. HPTLC Analysis.

Sr. No.	Parameter	Conditions used for Analysis	
		Filgrastim	Etanercept
1	Stationary phase	Silica gel 60 RP-18 F254sHPTLC plates	Silica gel 60 RP-18 F254sHPTLC plates
2.	Mobile phase	Water: n-butanol (7.5:2.5 v/v)	Isopropyl alcohol: water (6.5:4.5 v/v)
3.	Detection Wavelength	225 nm	222 nm
4.	Saturation time	15 min.	10 min
5.	Band width	6 mm	6 mm
6.	Retention Factor	0.45 ± 0.07	0.32 ± 0.03
7.	Densitogram		

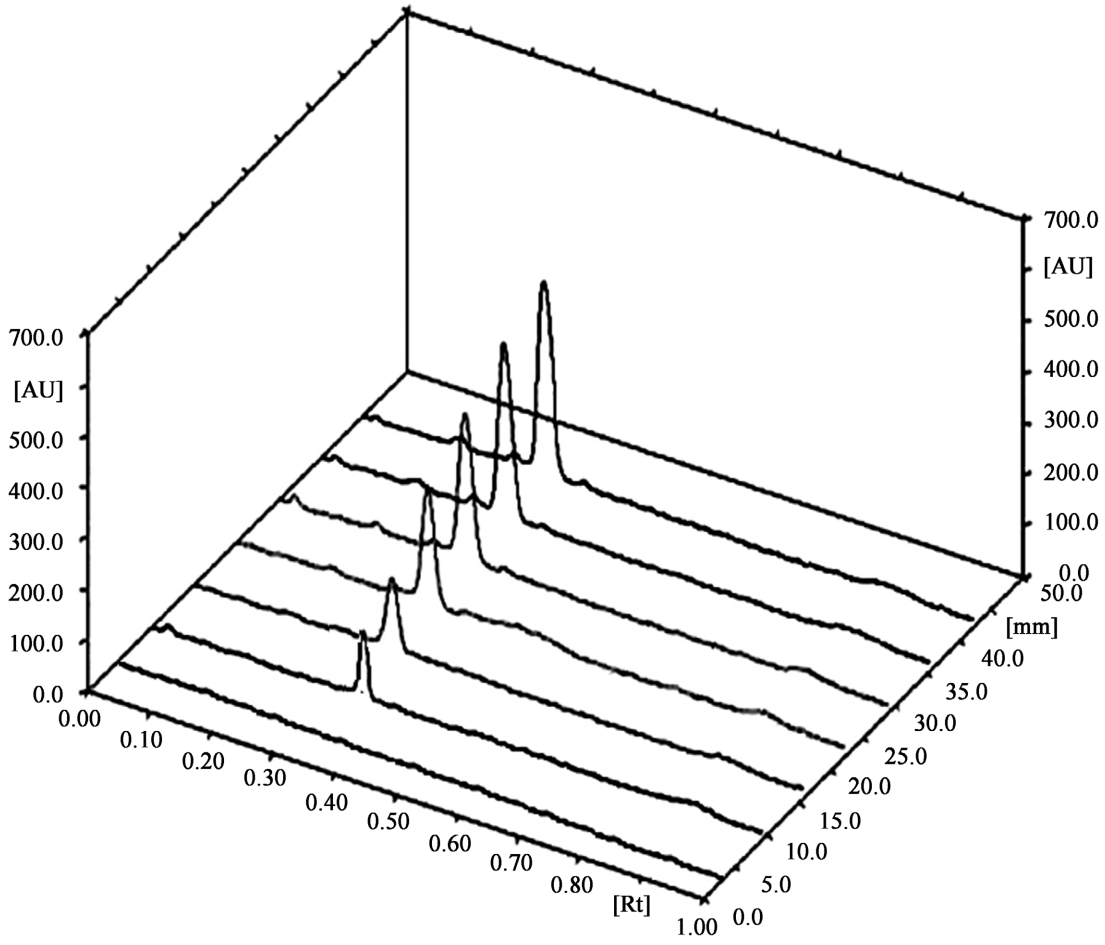


Figure 3. Densitogram of Etanercept.

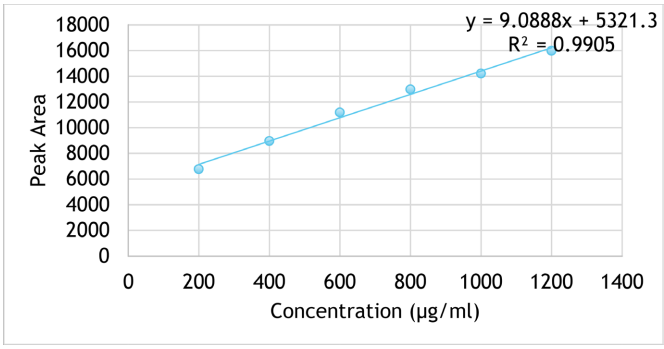


Figure 4. Calibration Graph of Etanercept.

determined by analyzing six concentrations over the concentration range 500 - 3000 ng/band to obtain calibration curve. The results were found to be linear with regression equation of y = 5.5173x + 2453.8 and r^2 value was found to be 0.9931. The densitogram and calibration curve are shown in **Figure 5** and **Figure 6**, respectively.

4.2.3. Precision

For both the drugs, the intra-day studies was analyzed using 3 replicates of 3

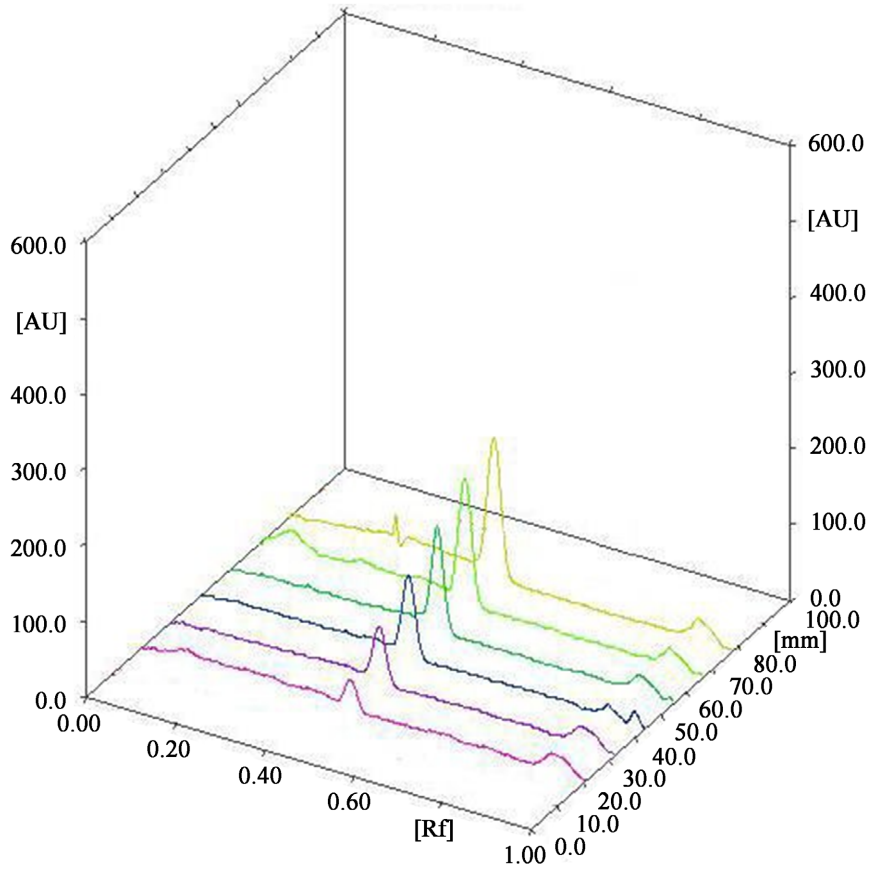


Figure 5. Densitogram of Filgrastim reference standard.

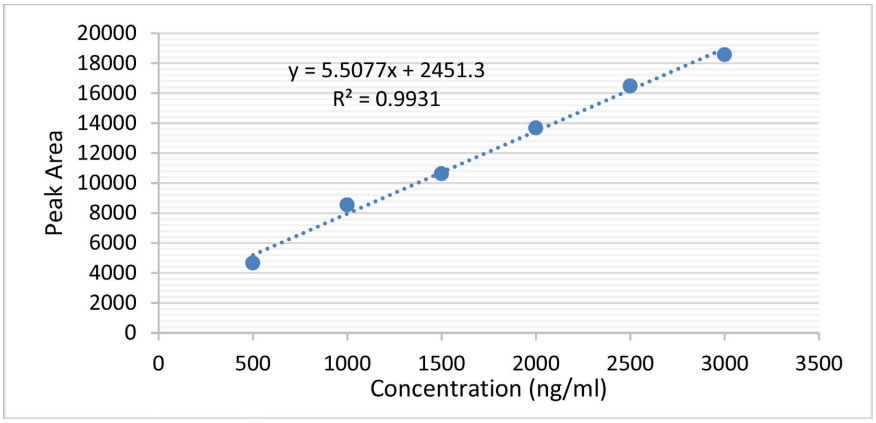


Figure 6. Calibration Graph of Filgrastim.

concentrations on the same day and percentage RSD were calculated. For the inter day variation studies, 3 replicates of 3 concentrations were analyzed on 3 consecutive days were calculated along with %RSD. For intraday precision and inter-day precision results obtained are shown in Table 2 and Table 3, respectively.

4.2.4. Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the sample solution, at three different levels 50%, 100% and

Table 2. Intraday and Interday Precision of Etanercept.

Concentration (µg/ml)	Intra-day Precision			Inter-day Precision		
	Average area	% Recovery	% RSD	Average area	% Recovery	% RSD
400	8549.1	98.926	1.381	8619.1	100.846	1.229
	8536.3	98.575		8536.3	98.575	
	8628.8	101.112		8549.1	98.926	
800	10,408.5	99.941	1.416	10,471.6	101.094	1.842
	10,317.8	98.283		10,317.8	98.283	
	10,471.6	98.926		10,508.5	101.769	
1200	12,279.3	98.575	1.575	12,318.6	101.143	1.173
	12,339.9	101.112		12,353.6	101.623	
	12,117.4	99.94		12,189.9	99.379	

Table 3. Intraday and Interday Precision of Filgrastim.

Concentration (ng/band)	Intraday Precision			Interday Precision		
	Average Area	% Recovery	%RSD	Average Area	% Recovery	%RSD
1000	8072.8	102.079	0.605	1010.785	101.078	0.168
	8031.1	101.322		1012.982	101.298	
	8005.9	100.864		1009.641	100.964	
1500	10,578.4	98.385	0.520	1484.327	98.955	1.261
	10,587.3	98.493		1511.765	100.784	
	10,655.9	99.323		1475.775	98.385	
2000	13,528.8	100.576	0.660	2023.641	101.182	0.392
	13,646.8	101.648		2008.823	100.441	
	13,664.2	101.806		2011.529	100.576	

150%. Basic concentration of sample chosen was 400 ng/band for etanercept and 1000 ng/ml for filgrastim. % Recovery was determined from linearity equation. The results obtained are represented in Table 4 and Table 5.

4.2.5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of Etanercept was found to be 33.381 ng/band and 101.153 ng/band, respectively whereas for filgrastim it was found to be 63.418 ng/band and 192.177 ng/band, respectively.

4.2.6. Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which scanning wavelength was altered. Time was also changed from spotting to development and development to scanning and the effect on the

Table 4. Recovery studies of Etanercept.

Level	Amount of sample taken (ng/band)	Amount of standard spiked (ng/band)	Area	% Recovery	Mean ± %RSD
50%	400	200	11,246.1	98.8	100.172 ± 1.196
			10,666.8	101.007	
			10,650.6	100.710	
100 %	400	400	11,977.6	100.992	99.779 ± 1.325
			12,916.4	998.371	
			12,033.4	99.975	
150%	400	600	14,046.4	99.865	99.222 ± 0.660
			13,989.9	99.245	
			13,627	98.556	

Table 5. Recovery studies of Filgrastim.

Level	Amount of sample taken (ng/band)	Amount of standard spiked (ng/band)	Area	Amount recovered (ng/band)	% recovery (Mean ± %RSD)
50%	1000	500	10,784.6	1513.218	100.390 ± 0.446
			10,735.2	1504.248	
			10,712.2	1500.071	
100%	1000	1000	13,648.2	2033.211	100.992 ± 1.227
			13,658.5	2035.081	
			13,417.1	1991.246	
150%	1000	1500	16,199.5	2496.494	100.564 ± 0.882
			16,433.6	2539.003	
			16,256.4	2506.826	

area were noted. It was found that method is robust. Table 6 and Table 7 represent the results obtained.

4.2.7. Assay

Etacept® 25 mg injection which is a lyophilized powder for reconstitution, contained 25 mg of Etanercept. Grafeel® pre filled syringes contained 300 µg/ml of Filgrastim. Analysis for both marketed formulations was carried out. Procedure was repeated for six times. Sample was spotted, scanned and area was recorded. Basic concentration of sample chosen was 400 ng/band for Etanercept and 1000 ng/ml for Filgrastim. Concentration and % recovery were determined from linear equation. Assay results obtained are shown in Table 8 and Table 9.

Table 6. Robustness data of Etanercept.

Sr. No.	Parameters	Variation	Concentration (ng/band)	%RSD
1.	Scanning wavelength	222± 1 nm	400	1.391 - 1.909
			800	0.427 - 0.695
			1200	1.624 - 1.993
2.	Time from application to development	(0, 30, 60 min.)	400	1.235 - 1.951
			800	0.427 - 1.183
			1200	1.993 - 2.009
3.	Time from development to scanning	(0, 30, 60 min.)	400	1.391 - 1.774
			800	0.427 - 0.937
			1200	1.369 - 1.993

Table 7. Robustness data of Filgrastim.

S. No.	Parameters	Variation	% RSD
1.	Time from application to development	0 min	1.465
		30 min	1.111
		60 min	0.759
2.	Time from development to scanning	0 min	1.477
		30 min	1.464
		60 min	0.879
3.	Scanning wavelength	225 nm	0.614
		224 nm	0.942
		226 nm	0.858

Table 8. Assay of marketed formulation (Etacept®).

Drug	Peak Area	Amount Recovered (µg/ml)	%Recovery	Mean ± %RSD
Etanercept	8619	403.372	100.843	99.814 ± 0.995
	8535.1	394.169	98.542	
	8549.5	395.749	98.937	
	8575.7	398.622	99.656	
	8584	399.533	99.883	
	8625.5	404.084	101.021	

5. Discussion

For analyzing biosimilar drugs requires state of the art technologies. Literature revealed research work showcasing the use of RP-HPLC, Peptide Mapping, circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy,

Table 9. Assay of marketed formulation (Grafeel®).

Sr. No	Peak area	Amount recovered (ng/band)	% Recovery
1.	7999.8	1007.534	100.753
2.	7947.6	998.055	99.806
3.	8003.2	1008.152	100.815
4.	7993.8	1006.445	100.644
5.	7983.4	1004.556	100.456
6.	8000.7	1007.698	100.770
Mean	7988.083	1005.407	100.541
SD	21.064	3.825	0.382
%RSD	0.264	0.380	0.380

(MALDI-TOF) mass spectrometry, liquid chromatography electrospray ionization (LC-ESI) mass spectrometry, fluorescence spectroscopy, sodium dodecyl sulfate polyacrylamide gel electrophoresis, high performance size-exclusion chromatography, dynamic light scattering (DLS), light obscuration, extrinsic fluorescence (Bis-ANS), far-UV circular dichroism (CD) spectroscopy, second derivative UV spectroscopy (UV), and enzyme-linked immunosorbent assay (ELISA) [13]-[16] for comparing innovator biopharmaceutical with marketed biosmilars. Batlovska-Borozanova *et al*., 2010; Dalmora *et al*., 2006; Qureshi *et al*., 2021 reported research work of analysis using RP-HPLC and UV for filgrastim. The LOD and LOQ values ranged from 0.1813 µg/ml to 24.35 µg/ml [17] [18] [19]. The LOD and LOQ values of the proposed method for Filgrastim were found to be 63.418 ng/band and 192.177 ng/band, for Etanercept 33.381 ng/band and 101.153 ng/band, respectively accentuating method's sensitivity. The selected biosimilars, filgrastim (18.8 KDa) and etanercept (150 KDa) contains 175 [3] and 934 amino acids [1], respectively and are protein in nature. Biller et.al, 2015 reported for separation of proteins by HPTLC, in which different proteins with molecular weights from 5777.5 Da (Insulin) to 66,432.9 Da (Bovine serum albumin) [20] were analyzed. In collation to sophisticated and complex techniques, the present research work offers the use of simple, easy and economical technique for analysis of biosimilar drugs.

6. Conclusion

Biosimilars structural characterization and comparison with that of innovator biopharmaceuticals requires the most advanced techniques. These techniques are neither cheaper nor less time consuming. The authors endeavored in developing the first simple, rapid and less expensive analytical technique using HPTLC for selected biosimilars, which is the need of the hour. The developed method can be used for comparing different biosimilars with the marketed formulations. It can also be used for the determination of presence of any post-translational

modifications, degradation products, etc. as complex biopharmaceuticals are prone to these changes and HPTLC offers easy detection of degradation products [21].

Conflicts of Interest

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

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