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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF TIGECYCLINE AND LINEZOLID IN BULK FORM.

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Abstract - This study presents the first reverse-phase high-performance liquid chromatography (RP-HPLC) method designed for the rapid and straightforward simultaneous detection and quantification of Tigecycline and Linezolid in both raw materials and pharmaceutical dosage forms. A selective, accurate, and reproducible HPLC method was developed and validated specifically for Tigecycline analysis in pharmaceutical products. The chromatographic separation was achieved using a reverse-phase Eclipse XDB C18 column (5 µm, 4.6 × 150 mm) with UV detection at 246 nm. The mobile phase consisted of a mixture of water, acetonitrile (ACN), and formic acid (90:10:0.1%) with a pH of 2.9, while the diluent was a combination of ACN, water, and methanol in the ratio of 20:50:30, applied in gradient elution mode at a flow rate of 1.0 mL/min. The developed method showed high sensitivity, specificity, accuracy, precision, and robustness. Linearity was confirmed with correlation coefficients (r^2) of 0.9999 (n = 5) for both drugs, validated over concentration ranges of 1–100 µg/mL for Tigecycline and 3–300 µg/mL for Linezolid. The repeatability (precision) was within 2% for both compounds. The limits of detection (LOD) were LOD and LOQ were -3.30 µg/mL and 9.98 µg/mL for Linezolid, while the limits of quantification (LOQ) were Linezolid the LOD and LOQ were - 1.71 µg/mL & LOQ - 5.20 µg/mL, respectively. The method's suitability was further demonstrated through accurate drug content determination in two commercial pharmaceutical formulations.

Introduction – Tigecycline is the first drug clinically available under the class of Glycylcyclines which are a new class of antibiotics derived from tetracycline. Tigecycline is a new glycylcycline with broad spectrum antibiotic activity. It is chemically (4S,4aS,5aR,12aS)-9-[2-(tert-butylamino)acetamido]-4,7-bis(dimethylamino)-3,10,12,12a tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a octahydrot etracene-2-carboxamide [1]. It was created due to the rising prevalence of antibiotic-resistant pathogens, including S. aureus, Acinetobacter, and E. coli. Due to its structural modifications, its therapeutic action has been widened to encompass both +ve and –ve gram bacteria and types resistant to several drugs. [3] It is considered as bacteriostatic which inhibits protein synthesis by inhibiting protein translation in bacteria by preventing incorporation of amino acid residues in to elongating peptide chains. It was first marketed under the brand name of TYGACIL

The present study aimed to evaluate the efficacies of LZD and TGC against potential pan-drug-resistant (PDR) bacteria Linezolid is the drug USFDA has approved linezolid for the treatment of infections caused by Gram-positive bacteria that are resistant to other antibiotics. Linezolid – a first synthetic oxazolidinones drugs in 1990s. [4] First approved for use in 2000, linezolid was the first commercially available 1, 3-oxazolidinone antibiotic. is active against Gram-positive bacteria that cause disease, including streptococci, vancomycin-resistant enterococci (VRE), and methicillin-resistant Staphylococcus aureus (MRSA). The safety and tolerability of linezolid are advantageous.

High-resolution structures of linezolid bound to the 50S ribosomal subunit show that it binds in a deep cleft that is surrounded by 23S rRNA nucleotides.[7] Linezolid is active against Most Gram-positive bacteria cause diseases, including streptococci, vancomycin-resistant enterococci, and methicillin-resistant Staphylococcus aureus [13]. The main indication of Linezolid is the treatment of severe infections caused by Gram-positive bacteria that are resistant to other antibiotics; it should not be used against bacteria that are sensitive to drugs with a narrower spectrum of activity, such as penicillin and cephalosporin. [10]. There is need to combine this drug to investigate potential synergistic or antagonistic effects, especially in the face of increasing antimicrobial resistance. To ensure accurate and reliable quantification of both drugs when they are present in the same sample, particularly in clinical or pharmaceutical settings. Clinical importance in treating multidrug-resistant infections and the potential for synergistic or complementary effects.This combination is used in the treatment of. Various bacterial infections includes intra abdominal infections as per reviewing of literature, we come to know that there no RP-HPLC study on this combination method has been reported till the time So, there is need to develop the RP-HPLC method for this combination. The innovation of this study lies in it being the first documented RP-HPLC method for the simultaneous detection of Tigecycline and Linezolid within a mixture. Unlike traditional methods that assess each compound separately, this combined detection approach improves efficiency and convenience by requiring only a single sample run. This not only conserves time and resources but also enhances accuracy and precision by reducing the variability associated with conducting multiple assays under different conditions. In addition to representing progress in the simultaneous determination of pharmaceutical compounds using HPLC techniques, this method offers a single, sensitive, and dependable analytical solution. It meets the growing need for effective analysis methods driven by the rising development of fixed-dose combinations and cost-effective generic medications.[2,3

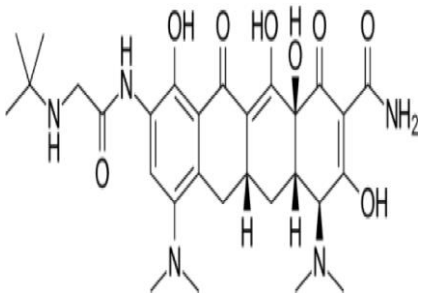


Fig.1 Chemical Structure of Tigecycline

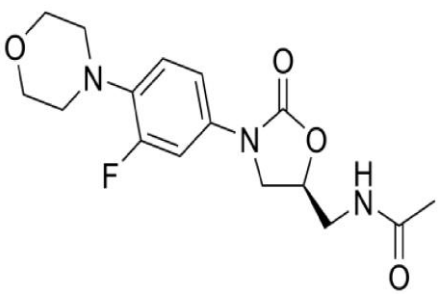


Fig.2 chemical structure of Linezolid

❖ Materials and Methods

Chemicals and Reagents:

Tigecycline Reference Standard was purchased from Dhamtech Pharma and Consultants Mumbai, India. And Linezolid drug was obtained from Optimus Drugs Private Ltd. Mumbai Acetonitrile HPLC grade was procured from Advent Performance Materials, High purity water was prepared with Elix -milli-Q system. Formic acid AR Grade Sigma.

Instrumentation : The HPLC instrument used Waters Alliance 2695 HPLC with PDA Detector 2996 infinity. Controlling the instrument parameters was through Empower software.

Selection of Wavelength - Absorption Spectra of A concentration of 25 µg/mL for both Tigecycline and Linezolid was analyzed within the wavelength range of 200–600 nm using water as the blank on a single-beam UV–visible spectrophotometer. The wavelengths suitable for the simultaneous estimation of both drugs were determined by identifying the intersection points on the overlaid UV absorption spectra of the two compounds. Both drugs obtained at this wavelength, Tigecycline at 250 nm and Linezolid at 251nm.

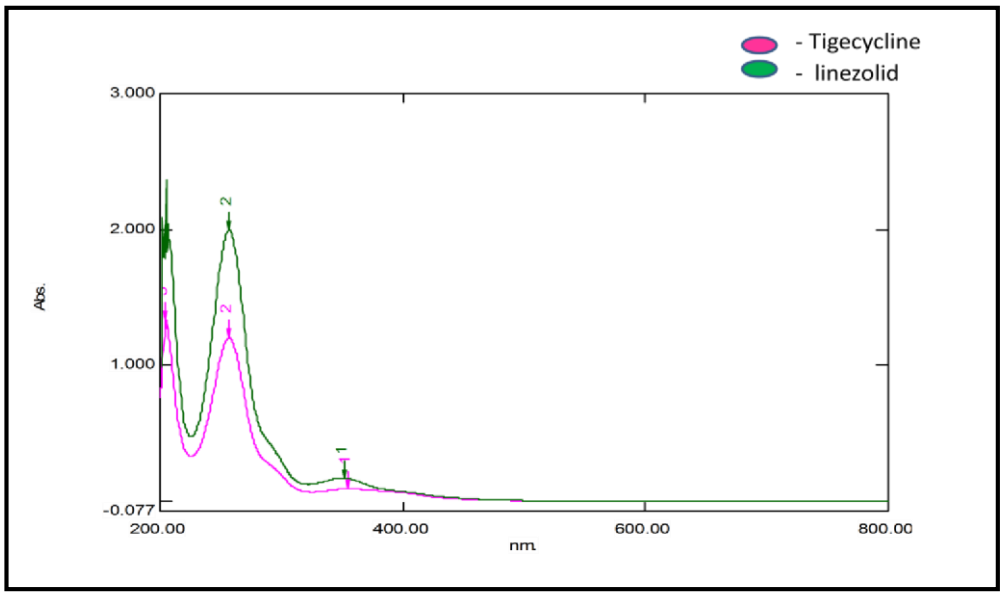


Fig. A UV – Vis absorption overlay spectra of Tigecycline and Linezolid

Development and optimization of the HPLC Method: Different combinations of mobile phases containing varying proportions of water, Formic acid, methanol, and acetonitrile were evaluated. To accomplish this, 2 distinct methods were experimentally investigated. Detection was carried out at wavelengths corresponding to the intersection points of the UV absorption spectra of the two drugs. After acquiring the chromatograms for each method, the peaks were analyzed, and the retention times (RT) were determined.

Chromatographic Conditions : The chromatographic separation of Tigecycline and Linezolid was carried out using an Eclipse XDB C18 column (5 µm, 4.6 × 150 mm) with a mobile phase composed of water, acetonitrile, and formic acid in the ratio of 90:10:0.1%, combined with methanol as the diluent. A gradient elution approach was employed. Prior to use, the mobile phase was filtered and degassed. The flow rate was maintained at 1.0 mL/min, and each sample analysis was completed within 16 minutes. UV detection was set at between 200 – 400 nm, and the column temperature was controlled at 27 ± 0.2 °C, with an injection volume of 10 µL. These chromatographic conditions were

optimized based on findings from the wavelength selection, instrumentation setup, and method development and optimization phases.

Preparation of mobile phase: A solvent mixture consisting of 83 mL of water and 17 mL of acetonitrile, prepared in a 50:50 ratio, was used for the auto sampler. Prior to use, both solvents were thoroughly degassed using an ultrasonic water bath for 5 minutes and subsequently filtered through a 0.22 µm membrane filter.

Preparation of Standard solution: A combined 1000 ppm standard solution of Linezolid and Tigecycline was prepared in a 50:50 ratio. Accurately weighed quantities of 100 mg each of Linezolid and Tigecycline were transferred to a 100 mL volumetric flask**.** Then diluent methanol was added, and the mixture was sonicated to ensure complete dissolution. The solution was then diluted to volume with methanol and mixed thoroughly. The final solution contained 1000 ppm for both drug.

Optimized method for Tigecycline and Linezolid	
Mobile phase -A	Water: ACN: FA (90:10:0.1%)
Mobile Phase- B	Methanol
Mobile phase	Gradient
Time	16min.
Flow	1.0ml/min
Injection volume	10µl
Wavelength	246nm (PDA)
Temp.	27°c
Diluent	ACN: Water: Methanol (20:50:30)
Column	Eclipse XDB C18 5µ (4.6*150) mm
Instrument	Water Alliance 1695 HPLC with PDA Detector 2995
Software	Empower 2

This is Generic Acidic method optimized for the Tigecycline + Linezolid in HPLC

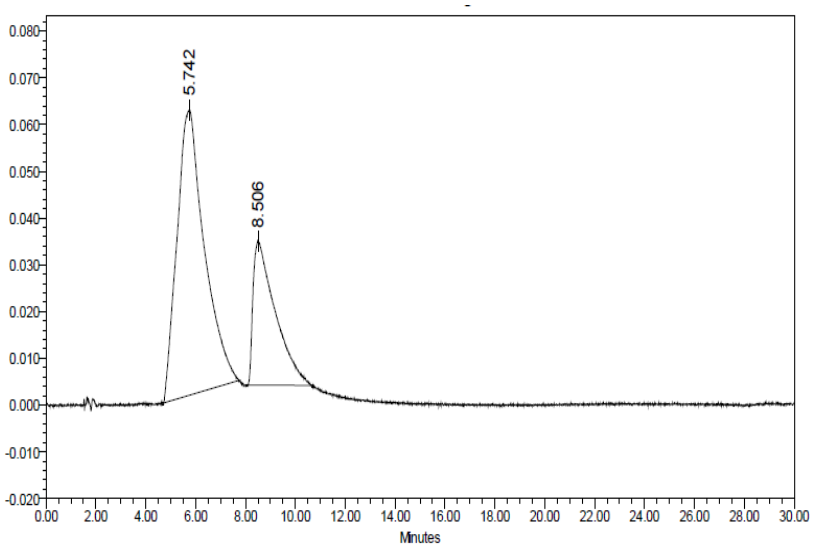


Fig. B Standard chromatogram for Linezolid + Tigecycline

Peak Results

	RT	Name	Area	% Area	USP Plate Count	USP Tailing	Resolution	USP Resolution
1	1.134	Tigecycline	6340005	36.98	1597.54	1.67		
2	1.710	Linezolid	10803343	63.02	4381.45	1.33	5.55	5.01

Table no. 1

Result and Discussion:

Method Validation : The HPLC method was validated by evaluation of the analytical parameters including specificity, linearity, precision, accuracy and robustness.

1.Linearity: Tigecycline reference solutions were prepared at concentrations of 100, 150, 200, 250 and 300µg/ml. Standard plots were constructed and linearity was evaluated statistically by linear regression analysis that was calculated by least squares regression. As discussed in the linearity, regression analysis results on the mean peaks area and concentrations data showed that the evaluated standard calibration curve is linear over the concentration range of 100 to 1µg/mL and 300 to 3µg/mL for Tigecycline and Linezolid, respectively. For Tigecycline y- mx+c m =27604 and c=61411, while for Linezolid m=25888 and c =19562. Values of the determination coefficients (R^2) obtained from linear regression analysis were 0.999 for Tigecycline and Linezolid 0.998.

Fig. a) Concentration at 100 ppm

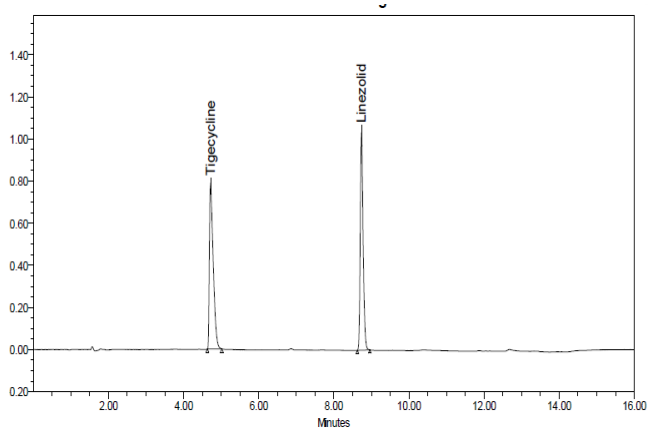
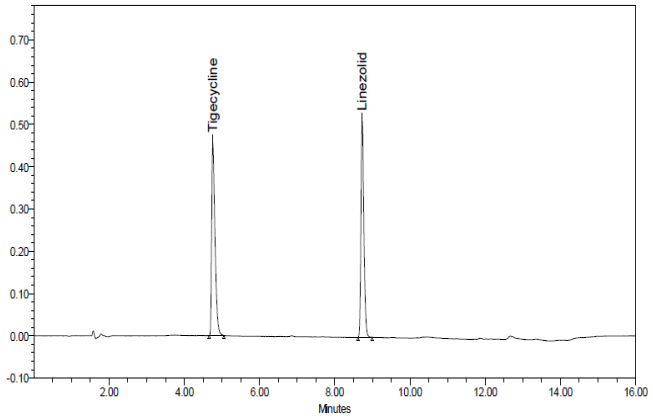


Fig.b) Concentration at 150 ppm



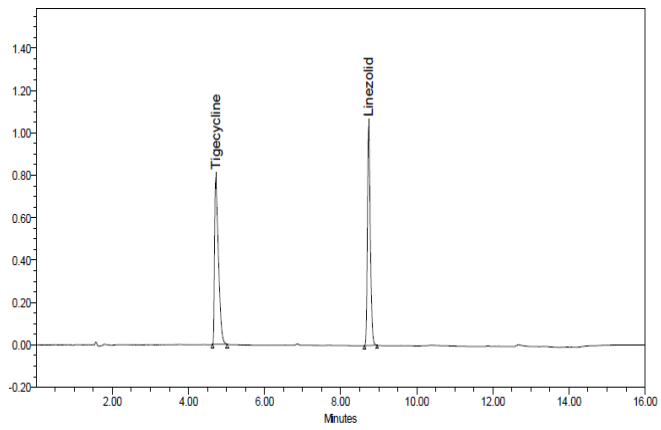


Fig.c) Concentration at 250 ppm

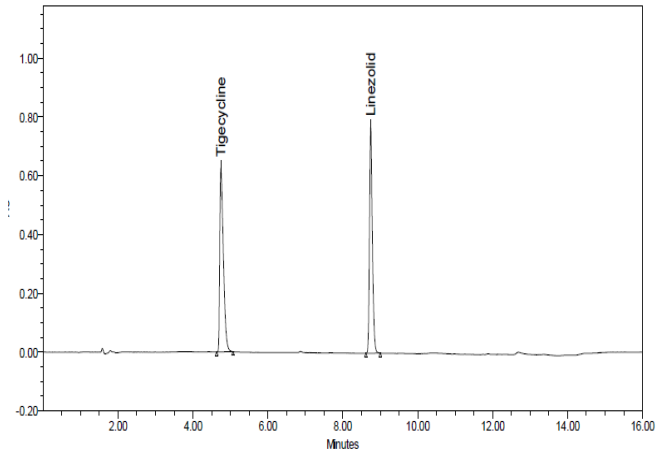


Fig.d) Concentration at 300 ppm

Linearity Graph :

Table no. 2

Linearity Plot	
Conce(mg/ml)	Area OF Tigecycline
100	2810731
150	4240732
200	5586289
250	6882566
300	8390839

Linearity Plot	
Conce (mg/ml)	Area of Linezolid
100	2748110
150	4113061
200	5432518
250	6591169
300	7980990

Table no. 3

2. Accuracy : The accuracy of the proposed method was assessed by recovery studies Tigecycline and Linezolid standard solution was spiked to sample solution at three concentration levels 100 %, 200 % & 300 %. Three replicates of each concentration level were prepared. These solutions were injected in HPLC and response was recorded. % Recovery was determined. The developed method was Acidic for both drug. Accuracy was expressed as the percentage of the reference standard recovered, reflecting the closeness between the measured concentrations and the actual true concentrations in the samples. The calculated recovery values for both Tigecycline and Linezolid were within the acceptable range, with recoveries ranging from 99.89 % to 101.897 ± 1.728%. These results confirm that the developed method is accurate.

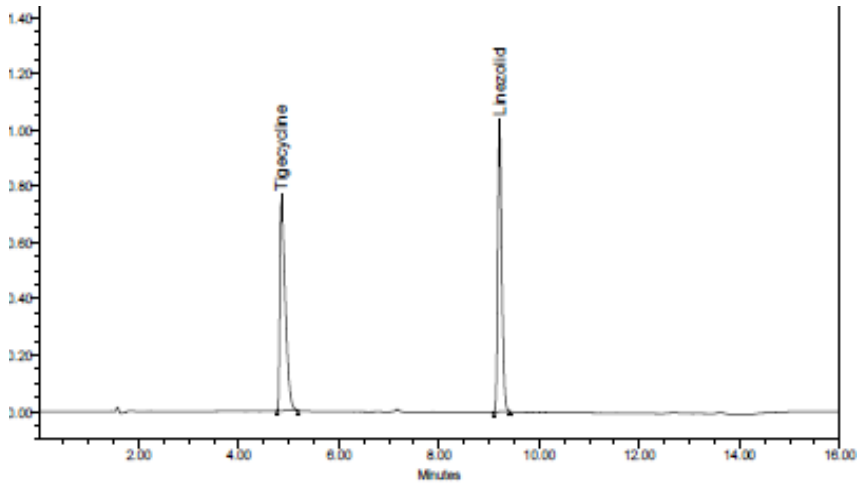


Fig. C Chromatogram for Tigecycline + Linezolid for Recovery

RT	Name	Area	%Area	USP Plate Count	USP Tailing	Resolution	USP Resolution
4.855	Tigecycline	5662666	50.73	8547.37	2.09	-	
9.209	Linezolid	5499943	49.27	66319.65	1.28	25.49	23.96

Table no.1

Table no.2 Observation table for Recovery:

Drugs	Spiked Conc.	Area	Calculated Conc.	% Recovery	SD	% RSD
Tigecycline + Linezolid	100	2798478 2736374	99.89	99.89%	4389.8	0.16%
	200	5662666 5499943	200.74	100.37%	11506.8	0.21%
	300	8366098 7964210	298.67	99.56%	28419.4	0.35%

3. Precision: The precision of the method was determined by repeatability (intra-day). In order to evaluate the repeatability of the methods, six sample solutions (200 µg/mL) were determined during the same day Precision was assessed by injecting six replicates of 10µg/ml Tigecycline and Linezolid standard solution, on the same day, and under the same experimental conditions. Peak area of six replicates of standard solution was obtained from chromatograms. % RSD was determined. Below show that intra-day and precision values at different nominated concentrations of the validated analytical method. Over all % RSD for intra-day precision for the proposed method were found to be less than 2%, which indicate the precision of the developed method.

Table no .1 Repeatability of Tigecycline

Injections	Area
1	5704612
2	5726597
3	5728762
4	5736722
5	5727328
6	5662666
Average	5714448
SD	27541.38
RSD %	0.48

Table no.2 Repeatability of Linezolid

Injections	Area
1	5515184
2	5531666
3	5532250
4	5531723
5	5531880
6	5499943
Average	5523774
SD	13451.58
RSD	0.24

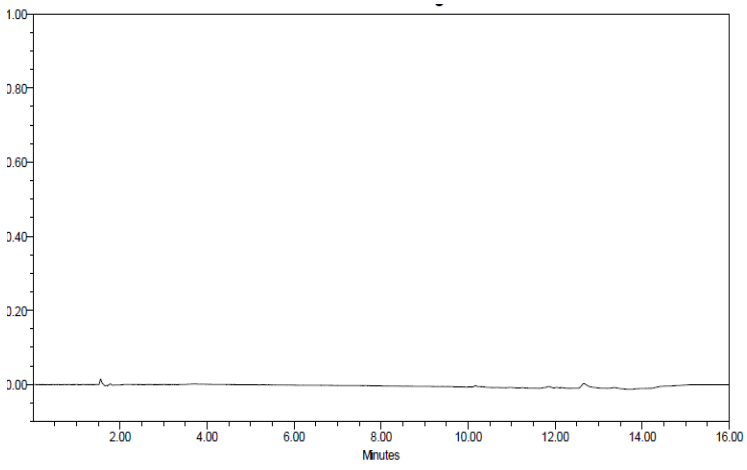


Fig. A Blank Chromatogram

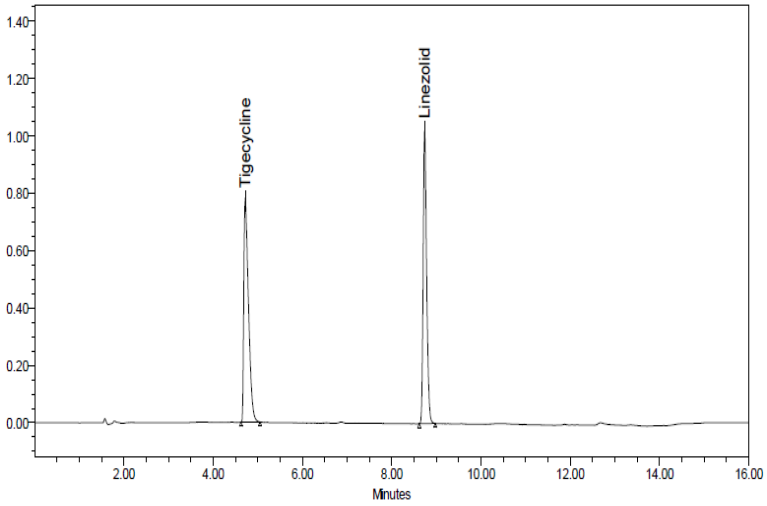


Fig.B Chromatogram TL at 200 ppm

	RT	Name	Area	% Area	USP Plate Count	USP Tailing	Resolution	USP Resolution
1	4.720	Tigecycline	5704612	50.84	8670.43	1.96		
2	8.739	Linezolid	5515184	49.16	60239.02	1.29	24.19	22.87

Table no.3

4. LOD and LOQ : Values of LOD and LOQ were evaluated using LOD = 3.3 *Sy*/*a*, LOQ = 10 *Sy*/*a*. For Tigecycline the LOD and LOQ were = 3.30 µg/mL and 9.98 µg/mL µg/mL, respectively. While for Linezolid the LOD and LOQ were = 1.71 µg/mL **& LOQ =** 5.20 µg/mL, respectively. LOD and LOQ are important parameters that should be established in studies to define the range of working concentrations of the drugs for their role in establishing the sensitivity of the method.

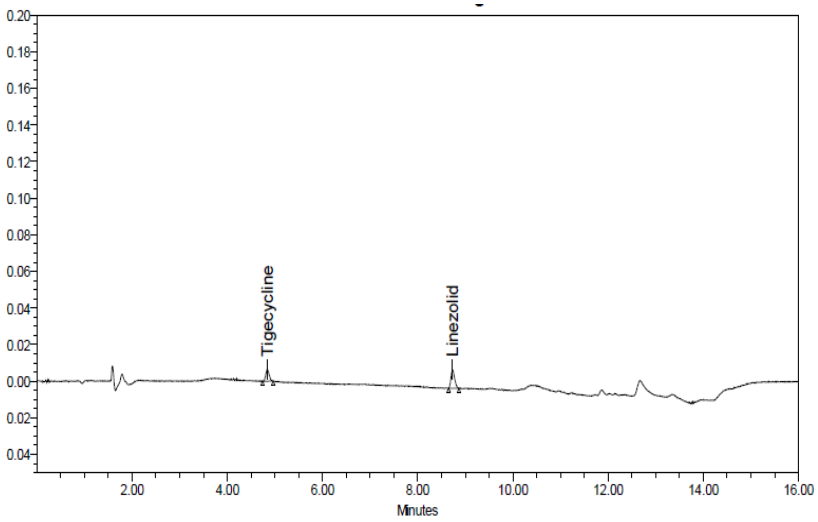


Fig. A Concentration at 1ppm (LOD)

Peak Results

	RT	Name	Area	% Area	USP Plate Count	USP Tailing	Resolution	USP Resolution
1	4.840	Tigecycline	31587	37.22	19193.71	1.15		
2	8.733	Linezolid	53271	62.78	57692.19	1.25	28.59	27.45

Table no. 1

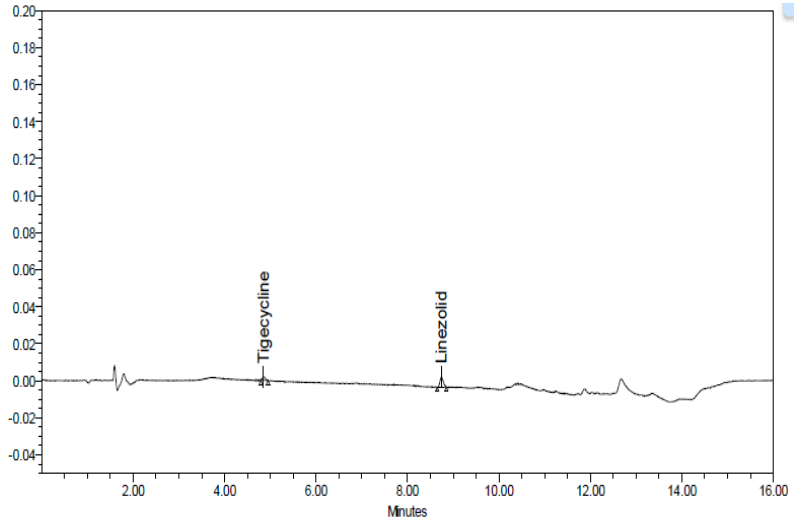


Fig.B Concentration at 2 ppm (LOQ)

Peak Results

	RT	Name	Area	% Area	USP Plate Count	USP Tailing	Resolution	USP Resolution
1	4.851	Tigecycline	9733	25.35	28250.70	1.30		
2	8.742	Linezolid	28660	74.65	49815.99	1.09	29.33	29.68

Table no. 2

5. Robustness:

The robustness of the proposed method was examined against small, deliberate variations of critical parameters such as the pH of the mobile phase, the flow rate and Temperature. Displays the results for the robustness of the validated analytical method. The flow Rate 0.9 -1.1ml/min. and Temperature effect was evaluated between. 25°c – 29°cThe results confirmed the robustness of the test for simultaneous determination of the two drugs since the observed variations were less than ±2 %. However, it was observed that the determination of Tigecycline is more sensitive to pH variation than that of Linezolid

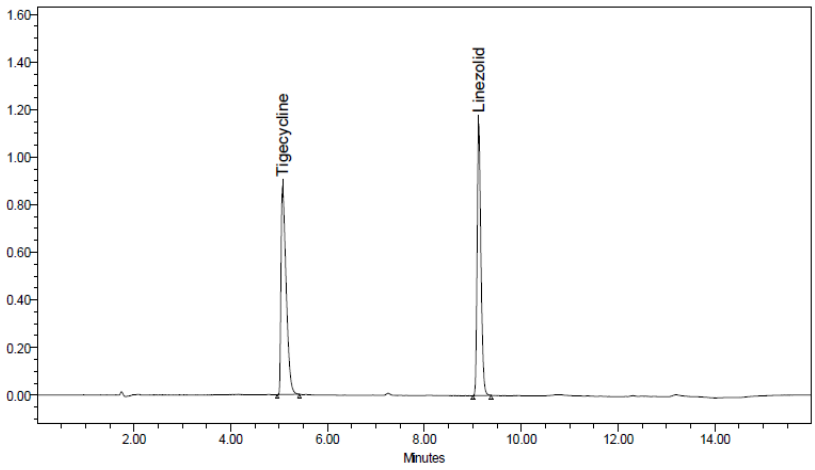


Fig. A Concentration at 0.9 flow rate

Calculations:

Change in flow rate(±0.1ml/min)	0.9ml/min	1.0ml/min	1.1ml/min
Average area	6433908	5586289	5117010
SD	66092	63711	82990
%RSD	1.02	1.14	1.62

Change in flow rate(±0.1ml/min)	0.9ml/min	1.0ml/min	1.1ml/min
Average area	6204111	5432518	4974144
SD	65889	67482	55856
%RSD	1.06	1.24	1.12

Table no.1

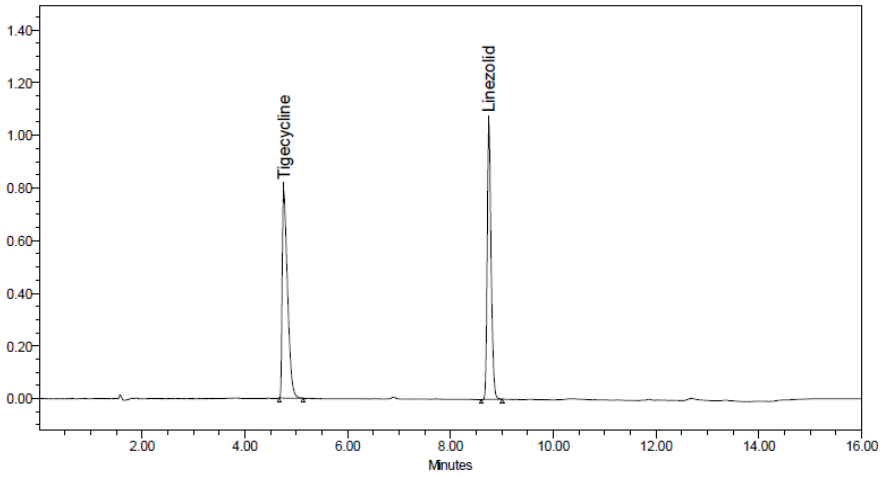


Fig.B Concentration at 25^{oc} Temp.

For Temperature :

change in column temp ±2^{0}C	29^{0}C	27^{0}C	25^{0}C
Average area	5698541	5683408	5726597
SD	61459	26185	43306
%RSD	1.07	0.46	0.75

change in column temp ±2^{0}C	29^{0}C	27^{0}C	25^{0}C
Average area	5516756	5496150	5531666
SD	33244	33850	38334
%RSD	0.60	0.61	0.69

Conclusions:

This work presented the first report of a rapid, simple, reverse-phase, high performance liquid chromatography method. The method was developed, optimized and validated for the isocratic separation and simultaneous determination of Tigecycline and Linezolid in their combined dosage form in a single mixture. Performed tests have shown that the method to be sensitive, selective, accurate, precise, and robust. The applicability of the developed method was demonstrated by successfully determining the drug content of two commercial pharmaceutical formulations. Finally, in contrast to conventional methods, attributes of this method provide the ability to accurately measure a great number of samples in relatively short time periods, making it suitable for quality control purposes in pharmaceutical industries and laboratories alike. Validation of the developed method was done as per International Conference on Harmonization (ICH) Q2R1 guidelines.

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