

Thermal Analysis and Validation of UV and Visible Spectrophotometric Methods for the Determination of New Antibiotic Tigecycline in Pharmaceutical Product

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Abstract This work has proposed the development and validation of ultraviolet (UV) and visible spectrophotometric methods for determination of tigecycline in lyophilized powder. In UV method tigecycline showed absorption maximum at 245 nm, in aqueous medium, where as in visible spectrophotometric method it reacts with copper acetate reagent, under acid conditions, forming a greenish coloured solution with absorption maximum at 378 nm. Thermogravimetric Analysis and Differential Scanning Calorimetry (TGA-DSC) techniques were studied to determine the thermal analysis of tigecycline. The methods were completely validated according to the International Conference on Harmonization (ICH) guidelines, showing good accuracy, precision, selectivity, robustness and linearity. Therefore the methods were found to be simple, economic and free of polluting reagents, being suitable for the analysis of the lyophilized powder formulation, contributing to improve the quality control and to assure its therapeutic efficacy.

Keywords Tigecycline, Spectrophotometric Methods, Thermal Analysis

1. Introduction

Tigecycline exhibits robust activity against bacterial isolates resistant to other antibiotic classes, including beta-lactams and fluoroquinolones, while resisting deactivation by most of the known tetracycline resistance mechanisms found in clinically significant bacteria[1]. This antibiotic has been evaluated as monotherapy for serious infections in human clinical trials as a result of its microbiological, pharmacodynamic and pharmacokinetic properties[2,3].

Tigecycline is structurally derived from minocycline by adding a tert-butyl-glycylamido side chain to carbon 9 of the D ring of the tetracycline backbone[4]. The Figure 1 depicts the structural formulas of tigecycline[5]. Chemically, tigecycline is [(4S, 4aS, 5aR, 12aS) -9- (2 - tert- Butylaminoacetyl-amino)-4,7-bis-dimethylamino-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydronaphthacene-2-carboxamide]. Its chemical formula is $C_{29}H_{39}N_5O_8$, and its molecular weight is 585.65 Daltons[6,7].

There are few methods described to analyze tigecycline and its metabolites in the literature. Four HPLC-UV assays for the determination of tigecycline in biological materials have been described in the literature [6, 8-11]. Besides

HPLC-UV, HPLC/MS/MS methods have also been employed to determination of tigecycline in biological fluids and tissues, as serum, urine, bronchoscopy and bronchoalveolar lavage, alveolar cells, gall bladder, bile, colon, bone, synovial fluid, lung and rat bone[6,10,12-14].

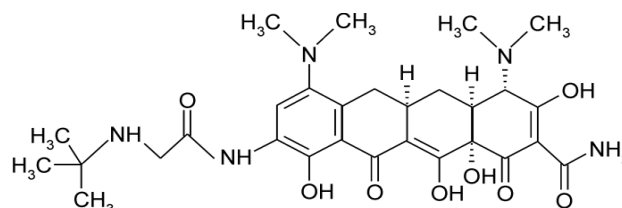


Figure 1. Chemical structure of tigecycline

The literature has reported analytical methods for determination of antibiotics such as chromatography, spectroscopy, spectrophotometry and microbiological assay. The microbiological assay is the most commonly used routine method. However, it is slow, often inaccurate, and subject to interference by other antibiotics[15]. Though modern methods of analysis (HPLC, GLC, NMR and Mass) for purity assay of any drug afford simplicity, speed, good specificity and excellent precision and accuracy, they involve sophisticated equipments, which are not in the reach of most laboratories and small-scale industries. Moreover, they pose problems of maintenance[16]. On the other hand, spectrophotometry has always provided analytical techniques characterized by instrumental simplicity, moderate cost and portability. These features make spectrophotomet-

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ric techniques particularly suitable for the determination of low concentrations of clinically important compounds[17].

The tigecycline is commercially available, but at the moment, there is no spectrophotometric method published for the quantitative analysis of this drug. Therefore, the aim of the present article was to develop and validate two simple and fast spectrophotometric methods for the quantitative analysis of tigecycline in lyophilized powder, contributing to improve the quality control and assuring the therapeutic efficacy. This paper reports procedures for the quantitation of the drug in pharmaceutical forms by visible and ultraviolet lights spectrophotometry, providing precise and accurate results validated by statistical analysis as well as being low cost methods.

2. Experimental

2.1. Chemical and Reagents

The tigecycline reference substance was purchased from Sequoia Research Products (Oxford, UK). Batches of Tygacil® (Wyeth, USA) lyophilized powder, containing 50 mg of tigecycline were obtained from commercial sources within their shelf-life period. Copper acetate was purchased from Merck (Darmstadt, Germany) and sodium acetate from Synth (Diadema, Brazil). All chemicals used were of pharmaceutical or special analytical-grade. For all of the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Massachusetts, USA).

The placebo solution was prepared in the laboratory by addition of appropriate amount of pharmaceutical grade excipient monohydrate lactose in purified water.

The acetate buffer pH 3.0 was prepared by dissolving 4.0 g of anhydrous sodium acetate in 800 mL of water. Sufficient amount of glacial acetic acid (about 130 mL) was added to adjust pH. The solution was transferred into a 1000 mL volumetric flask and diluted to 1000 mL with water.

To prepare the copper acetate solution, 5.0 g of copper acetate were weighed and transferred into a 100 mL volumetric flask, 80 mL of water was added and the solution was placed in ultrasonic bath for 30 minutes, then the volume was completed with water.

2.2. Apparatus

All absorbance measurements were performed on a UV-Vis Recording Spectrophotometer UV-mini 1240 (Shimadzu®, Kyoto, Japan), using 1.0 cm quartz cells. To adjust of buffer pH, in the visible method, a digital pHmeter B474 (Micronal®, São Paulo, Brazil) was used. TGA (thermogravimetric analysis) curves were obtained with a SDT-2960 (TA Instruments) thermal analyzer apparatus using flowing nitrogen 100 mL min⁻¹, temperature range 25–1000°C, at a heating rate of 10°C min⁻¹. The DSC curves were obtained with a DSC-2910 (TA Instruments) equipment using flowing nitrogen 25 mL min⁻¹. The tigecycline

mass applied was 4.00 ± 0.4 mg to the two thermal analysis.

2.3. Preparation of Reference Substance Solution

2.3.1. UV Method

The stock solution was daily prepared by weighing accurately, 5 mg of tigecycline reference substance, transferred to 100 mL volumetric flask and diluted to volume with water, obtaining a concentration of 50 µg mL⁻¹ of tigecycline.

2.3.2. Visible Method

The stock solution was daily prepared by weighing accurately, 5 mg of tigecycline reference substance, transferred to 25 mL volumetric flask and diluted to volume with water, obtaining a concentration of 200 µg mL⁻¹ of tigecycline.

2.4. Preparation of Sample Solutions

2.4.1. UV Method

To prepare the sample solution, vials containing 50 mg of tigecycline were accurately weighed and mixed. An appropriated amount was transferred into an individual 50 mL volumetric flask and diluted to volume with water. The final concentration of 50 µg mL⁻¹ of the active pharmaceutical ingredient was daily prepared, diluted to an appropriate concentration with water, analyzed and the amount of the drug calculated against the reference substance.

2.4.2. Visible Method

To prepare the sample solution, vials containing 50 mg of tigecycline were accurately weighed and mixed. An appropriated amount was daily transferred into an individual 25 mL volumetric flask and diluted to volume with water, obtaining final concentration of 200 µg mL⁻¹ of the active pharmaceutical ingredient. Aliquots of this solution were transferred to volumetric flask to reaction with copper acetate, in acid pH, and diluted to an appropriate concentration with water, analyzed and the amount of the drug calculated against the reference substance.

2.5. Spectrophotometric Measurements

2.5.1. UV Method

Aliquots of reference substance and sample solutions were transferred into a 10 mL volumetric flasks and the volume was brought to the volume with water and the absorbance was measured at 245 nm against a reagent blank prepared simultaneously.

2.5.2. Visible Method

Aliquots of reference substance and sample solutions were transferred into a 10 mL volumetric flasks, followed by addition of 1 mL of acetate buffer, then 1 mL of 5 % (w/v) copper acetate solution and the volume was brought to the volume with water and the absorbance was measured at 378 nm against a reagent blank prepared simultaneously.

2.6. Validation of the RP-LC Method

The methods were validated using samples of pharmaceutical formulations with the label claim of 50 mg by determinations of the following parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness, following the International Conference on Harmonization (ICH) guidelines[18].

2.6.1. Specificity

Selectivity was evaluated by analysis of the placebo solutions and the tigecycline working standard solution at the concentration of 16 and 22 $\mu\text{g mL}^{-1}$, to ultraviolet and visible methods, respectively. The placebo solution containing the excipient monohydrate lactose of the pharmaceutical formulation was prepared for this study. It was treated in the same manner as the commercial samples.

2.6.2. Linearity and Range

The linearity was determined by constructing three independent analytical curves, each one with seven reference substance concentrations of tigecycline in triplicate, in the range of 10-22 and 10-34 $\mu\text{g mL}^{-1}$, to ultraviolet and visible methods, respectively. The absorbance values were plotted against the respective concentrations of tigecycline to obtain the analytical curve. The results were subjected to regression analysis by a least-squares method to calculate calibration equation and determination coefficient.

2.6.3. Precision and Accuracy

The precision of the method was determined by the repeatability and the intermediate precision. Repeatability was examined by six evaluations of the same concentration sample of tigecycline, on the same day, and under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days), and also by other analysts performing the analysis in the same laboratory (between-analysts). In the ultraviolet method, the accuracy was evaluated by applying the proposed method to the analysis of known amounts of the reference substance added to sample solution containing 10 $\mu\text{g mL}^{-1}$ of tigecycline, to obtain solutions at concentrations of 12.8; 16 and 19.2 $\mu\text{g mL}^{-1}$, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively. In the visible method, solutions at concentrations of 17.6; 22 and 26.4 $\mu\text{g mL}^{-1}$ were obtained by addition of known amounts of the reference substance to sample solution containing 14 $\mu\text{g mL}^{-1}$ of tigecycline. The accuracy of the methods was calculated as the percentage of the drug recovered from the sample.

2.6.4. Limits of Detection and Quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated, as defined by ICH (2005)[18], using the mean values of three independent analytical curves, determined by a linear-regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respec-

tively, were multiplied by the ratio from the standard deviation of the intercept and the slope.

2.6.5. Robustness

Three sample solutions were prepared and analyzed using the established conditions and by variation of the following analytical parameters: wavelength, volume of copper solution added (0.8–1.2 mL) and buffer pH (2.5-3.5). Tigecycline contents and R.S.D. were determined for each condition.

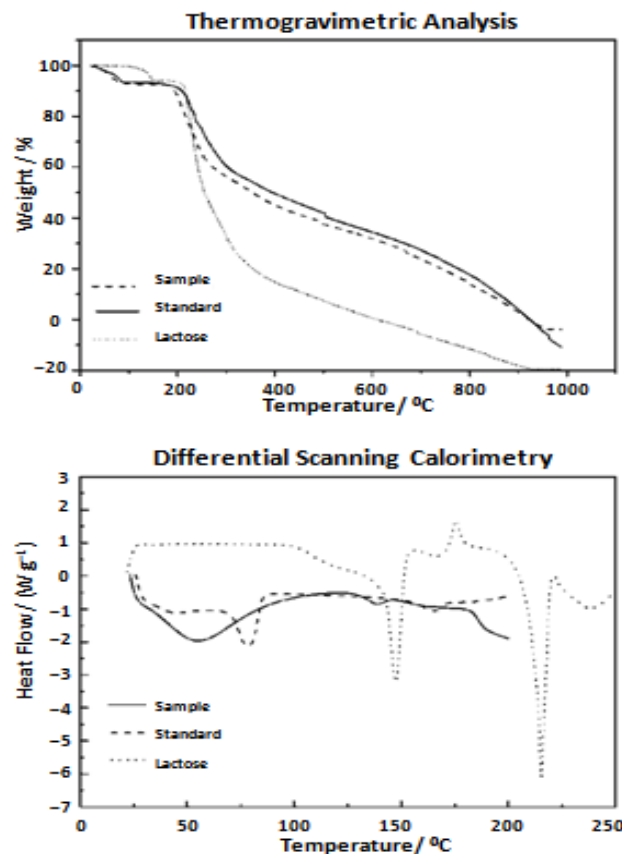


Figure 2. Thermal analysis of tigecycline

3. Results and Discussion

3.1. Thermal Analysis

The thermal analysis of the tigecycline standard and pharmaceutical product and the excipient monohydrate lactose was studied by Thermogravimetric Analysis- Differential Scanning Calorimetry (TGA-DSC) techniques. The TGA-DSC curves of tigecycline and lactose are shown in Figure 2. The tigecycline was heated from 25°C up to 1000°C. The TGA curves showed that the tigecycline standard was more thermally stable than the pharmaceutical product, since that the standard started its decomposition at 188.6°C and the sample at 175.3°C. The loss of the water happened between 33 and 127°C to sample and between 41 and 99°C to standard. The decomposition of lactose was observed at 198.4°C and the water loss happened between 89 and 161°C. In the DSC

curve of tigecycline sample, a water loss was observed followed by decomposition, indicating its amorphous state, and a melting point peak was not observed. To standard, the DSC curve showed water loss and after a melting point endothermic peak at 78.3°C, followed by its decomposition. The DSC curve of lactose showed water loss and after a melting point endothermic peak at 147.2°C, followed by crystallization verified in the exothermic peak at 175.6°C. It was observed a melting point endothermic peak at 215.1°C and enthalpy of 71.1 J g⁻¹. After, an additional endothermic peak at 236.4°C was formed, related to thermal decomposition of the melted compound.

3.2. Ultraviolet Method

The proposed UV method allows a rapid and economical quantitation of tigecycline in lyophilized powder without any time-consuming sample preparation. Moreover, the spectrophotometric methods involve simple instrumentation compared with other instrumental techniques. The absorption spectra of tigecycline in aqueous solution is shown in Figure 3. The λ max was found to be 245 nm. This wavelength was used for all measurements.

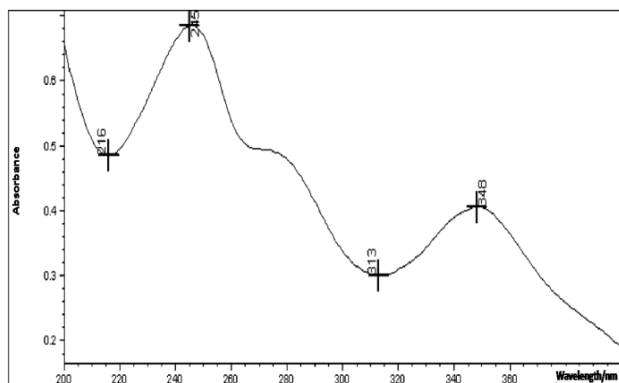


Figure 3. Ultraviolet absorption spectra of tigecycline in aqueous solution

For more accurate analysis, Ringbom curve was constructed and the linear range was observed. Calibration curves were constructed in the range of expected concentrations (10-22 $\mu\text{g mL}^{-1}$). Beer's law is obeyed over this concentration range. The value of the determination coefficient calculated ($r^2 = 0.9998$, $y = 0.0354x + 0.0003$, where, x is concentration and y is the absorbance value) indicated the linearity of the analytical curve for the method (Table 1).

Table 1. Analytical Parameters for the Determination of Tigecycline Using the Proposed Methods

	UV- spectrophotometry	Visible- spectro-photometry
Analytical wavelength/nm	245	378
Linearity range/ ($\mu\text{g mL}^{-1}$)	10-22	10-34
Regression equation ($A=aC+b$)		
Slope (a)	0.0354	0.0249
Intercept (b)	0.0003	0.0043
LOQ	0.33	0.29
LOQ	1.00	0.89
Determination coefficient	0.9998	0.9997

The LOD and LOQ were found to be 0.33 and 1.00 $\mu\text{g mL}^{-1}$, respectively. According to ANOVA there are linear regression and no deviation from linearity. The experimental values obtained for the determination of tigecycline in samples, indicated a satisfactory intra-day variability (R.S.D. of 1.12%) and intermediate precision (R.S.D. < 1.03%) (Table 2).

Table 2. Inter-day and between-analysts precision data of UV and visible methods for tigecycline in pharmaceutical product

Method	Sample	Inter-Day RSD ^a / (%)	Between-Analysts RSD ^a / (%)
UV-spectrophotometry	1	1.01	1.03
	2	0.22	0.52
Visible-spectrophotometry	1	0.73	1.83
	2	0.36	0.11

^a Relative standard deviation

A good accuracy of the method was verified with a mean recovery of 100.84% (Table 3). Variations in the wavelength to method robustness evaluation showed R.S.D. < 0.27% (Table 4).

Table 3. Accuracy of UV and Visible Spectrophotometric Methods for Tigecycline in Pharmaceutical Products

Method	Sample concentration	Added concentration/ ($\mu\text{g mL}^{-1}$)	% Recovery ^a $\pm$ RSD ^b / (%)
UV-spectrophotometry	10	2.8	100.32 $\pm$ 0.82
	10	6.0	100.78 $\pm$ 0.52
	10	9.2	101.42 $\pm$ 1.55
Visible-spectrophotometry	14	3.6	99.69 $\pm$ 0.94
	14	8.0	99.34 $\pm$ 0.71
	14	12.4	98.27 $\pm$ 1.77

^aMean of three replicates; ^bRelative standard deviation.

3.3. Visible Method

In the determination of the method conditions different buffer pH and copper concentration were evaluated. Variable amounts of copper solution (0.1-2 mL) were studied with fixed pH and tigecycline concentration. The volume of 1 mL of copper solution showed major absorbance values (Figure 4).

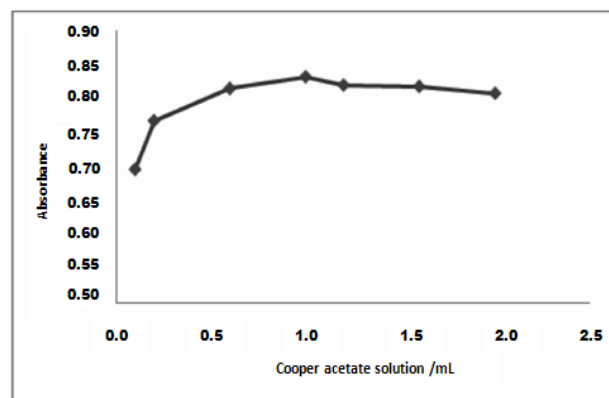


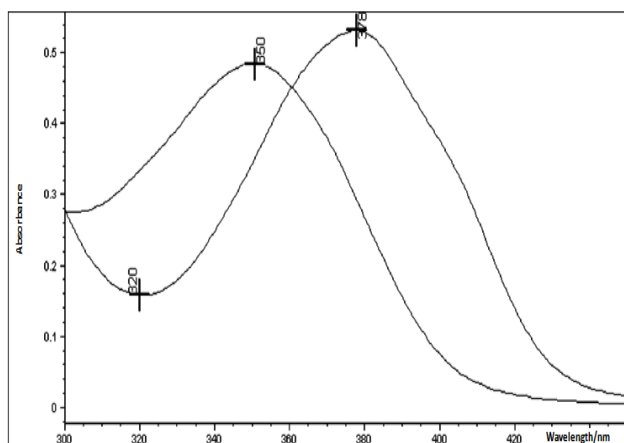
Figure 4. Effect of copper solution volume on the absorbance of 34 $\mu\text{g mL}^{-1}$ of tigecycline

Table 4. Robustness Evaluation of UV and Visible Spectrophotometric Methods to Determination of Tigecycline in Pharmaceutical Products

Method	Variable	Investigated range	Tigecycline ^a / (%)	RSD ^b / (%)
UV-spectrophotometry	Wavelength/nm	243	108.96	0.10
		245	109.16	
		247	109.12	
Visible-spectrophotometry	Wavelength/nm	376	110.53	0.13
		378	110.64	
		380	110.82	
	Buffer pH	2.5	110.24	0.28
		3.0	110.64	
		3.5	110.04	
	Copper solution/ mL	0.8	110.99	1.04
		1.0	110.64	
		1.2	108.85	

^aMean of three replicates; ^bRelative standard deviation.

After copper concentration determination, the following buffers and pHs were evaluated: sodium acetate (pH 3.0), ammonium acetate (pH 5.0), potassium phosphate (pH 7.5) and Tris (pH 8.6). Besides, the influence of temperature and light in the absorbance was determined. The best results were obtained with 1 mL of copper solution and sodium acetate (pH 3.0) buffer. The pH show low influence in the reaction, with similar absorbances at pHs 3.0, 5.0 and 8.6, however when the phosphate buffer was used, turbidity was observed, that may be related to copper precipitation occurring as oxides and hydroxides, at pH levels higher than 5 or 6[19]. The acid pH was chosen because in this pH tigecycline is incolor and there is not interference in the color of the reaction product. Then, the green color observed is from reaction product and not from mixture of yellow tigecycline and blue copper acetate solutions. A decrease in absorbance was observed after 15 min at 40°C. The reaction was stable for 30 minutes, in the presence or not of light. At pH 3.0, a maximum absorbance wavelength of 350 nm to tigecycline in aqueous solution was observed and, after addition of copper solution, this maximum increased to 378 nm, indicating the occurrence of the reaction (Figure 5).

**Figure 5.** Absorption spectrum of tigecycline in the presence ($\lambda_{\max} = 378$ nm) and absence ($\lambda_{\max} = 350$ nm) of copper solution at pH 3.0

The reaction mechanism between tigecycline and copper was not elucidated, since this work is the first study that

applies this reaction. Although, for tetracyclines analogs it is shown that the first donor group to bind copper is the O3 atom, which starts deprotonating at relatively low pH. When the pH is raised chelation taking place at the O10-O12 system as the OH12 group dissociates. The ultimate ligand deprotonation at the C4 dimethylammonium group leads to the chelate formation, with a new change in the binding mode in favor of the N4-OH12, donor set[20].

The analytical curves constructed for tigecycline were found to be linear in the 10-34 $\mu\text{g mL}^{-1}$ range. The value of the determination coefficient calculated ($r^2 = 0.9997$, $y = 0.0249x - 0.0043$, where, x is concentration and y is the absorbance) indicated the linearity of the analytical curve for the method. The precision, evaluated as the repeatability of the method, was studied by calculating the relative standard deviation (RSD) for six determinations of the 22 $\mu\text{g mL}^{-1}$, performed on the same day and under the same experimental conditions. The obtained RSD value was 0.34%. The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day); the mean values obtained were 110.79 and 119.47% with RSD 0.73 and 0.36%, respectively. The between analysts precision was determined by calculating the mean values and the RSD for the analysis of two samples of the pharmaceutical formulation by two analysts; the mean values were found to be 110.23 and 119.53% with RSD 1.83 and 0.11%, respectively. The results are shown in Table 2. The accuracy was assessed from three replicate determinations of three different solutions containing 17.6, 22 and 26.4 $\mu\text{g mL}^{-1}$. The absolute means obtained for tigecycline are shown in Table 3 with a mean value of 99.10%, demonstrating that the method is accurate within the desired range. For calculating of the LOD and LOQ, a calibration equation, $y = 30773.77x + 62280.15$, was generated by using the mean values of the three independent analytical curves. The LOD and LOQ calculated were 0.29 and 0.89 $\mu\text{g mL}^{-1}$, respectively. The results and the experimental range of the selected variables evaluated in the robustness assessment are given in Table 4.

Table 5. Determination of tigecycline in pharmaceutical products by the UV and visible methods

	Label claim		Experimental amount		
		Tigecycline	Tigecycline ^a		
Method	Sample	mg per vial	mg	Recov-ery/ (%)	RSD ^b / (%)
UV-spectrophotometry	1	50	55.57	111.14	0.83
	2	50	61.21	122.41	0.41
Visible-spectrophotometry	1	50	55.19	110.37	0.54
	2	50	61.48	122.95	1.02

^aMean of three replicates; ^bRelative standard deviation.

3.4. Methods Application

The proposed spectrophotometric methods were applied for the determination of tigecycline in lyophilized powder, without prior separation of the excipient of the formulation,

showing the applicability of the method for quality control analysis (Table 5). The results demonstrated elevated tigecycline content in the samples, however these results are not considerate irregulars since official reference standard and specifications for tigecycline in lyophilized powder are not established yet.

4. Conclusions

In this work, analytical ultraviolet and visible spectrophotometric methods were developed for quantitative determination of tigecycline in lyophilized powder. Its advantages over other existing methods are its simplicity, fastness and low-cost conditions. Moreover, it does not use polluting reagents. All validation parameters were found to be highly satisfactory, indicating linearity, selectivity, precision, accuracy, robustness and adequate detection and quantification limits. The method, therefore, can be easily applied in routine quality control laboratories.

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