Development and Validation of A Novel HPLC Method for Quantification of Tigecycline in Bulk and Nanoparticle Based Suspension

GEETHA M1*, PRAKASH RAO B2, RAJENDRA CE3, CHALUVARAJU KC4, AND MANJULA BP5
1*, 4, 5 Government College of Pharmacy, Rajiv Gandhi University of Health sciences, Bengaluru - 560 027, India
2 Karnataka College of Pharmacy, Rajiv Gandhi University of Health Sciences, Bengaluru - 560 064, India.
3 Drugs Testing Laboratory, Bengaluru - 560 001, India.

ABSTRACT

A novel and simple HPLC method was developed and validated for the determination of tigecycline in bulk and nanoparticle based suspension. The optimal separation was achieved with in 15 min with a column RP - C18 5μ (250×4.6 mm) LiChrospher® 100 and a mobile phase containing buffer pH 6.7 and acetonitrile in the ratio of 72:28. Detection wavelength was set at 248 nm. Good linearity and recovery were achieved over the range of standard curve. The relative standard deviations for repeatability, intra- and inter-day precision were less than 2 %. The method was suitably validated with respect to specificity, linearity, limit of detection and quantification, accuracy, precision and robustness and was successfully applied for the determination of tigecycline in nanoparticle based suspension.

Key words: Tigecycline, Nanoparticle, HPLC, Validation,

INTRODUCTION

Across the several years, antimicrobial resistance is prevailing predominantly specially among various gram-positive organisms such as drug-resistant pneumococci methicillin-resistant staphylococci, vancomycin-resistant enterococci. Only few antimicrobials are available for use in these resistant infections. Tetracyclines are one among these, active against both gram-positive and gram-negative bacteria as well as atypical organisms. Tetracycline usage for treating bacterial infections has been limited in recent years because of the emergence of resistant organisms with efflux and ribosomal protection mechanisms of resistance [1-3]. A tetracycline analogue, novel glycycline antibiotic has been developed to combat the above antimicrobial resistance mechanisms. Tigecycline is superior to quinolones, β-lactams and tetracyclines in treating drug resistant infections. Moreover, tigecycline causes minimal organ toxicity, and dosage adjustment is not necessary in most patient populations [4-5]. Chemically, tigecycline is [4S-(4α, 4aα, 12αα)-4,7-Bis (dimethylamino) – 9 – [2-(1,1-diethylethyl) acetylaminol]- 1,4,4a,5,5a,6,11,12α-octahydro-3,10,12,12α-tetrahydroxy-1,11-dioxo-2-naphthacene carboxamide [6].

Regarding its mechanism of action, tigecycline enters bacterial cells through energy dependent pathways or by passive diffusion, and reversibly binds to the 30S subunit of the ribosome. It acts by blocking the incorporation of transfer RNA into the A site of the ribosome, thus inhibiting protein synthesis [7]. Tigecycline, the 9-t-butyl glycyldiamido derivative of minocycline, is the first marketed compound of this new class of antibiotics. It was also found to act faster against plasmodium than any of the other antibiotics tested [8].

Based on literature survey, many analytical methods have been reported for the estimation of tigecycline in bulk, pharmaceutical dosage forms and biological fluids, which include, liquid chromatography[9], LC/MS/MS[10], high-performance liquid chromatographic UV detection technique[11], liquid chromatography/tandem mass spectrometry [12], online solid phase extraction (SPE) HPLC [13], thermoanalysis and validation of UV and visible spectrophotometric methods[14], stability indicating RP-HPLC method development and its validation[15], Stability
indicating HPLC determination[16], photo-chemically induced fluorescence determination by a stopped-flow multicommutated flow analysis assembly[17], area under the curve spectrophotometric method[18], development and validation of RP-HPLC pre-column derivatisation for the trace level determination of tertbutylamine in tigecycline drug substance[19], validation of a stability indicating RP-LC method[20], turbidometric assay for potency evaluation[21].

The aim of this study was to develop a new HPLC method and to exhibit validation strategies for the analysis of tigecycline in nanoparticle based suspension. In view of this, influence of mobile phase ratio, buffer concentration and flow rate was systematically investigated and method validation studies were performed.

Materials and Methods

Materials

Reference standard and Tigecycline drug sample were obtained as gift samples from Aurobindo Laboratories Hyderabad, India. High performance liquid chromatography grade acetonitrile and all other analytical grade reagents were purchased from Spectrochem private limited, India. HPLC grade water was prepared using Milli-Q water purification system. Class A glassware is used throughout the experiment.

Instrumentation

The experiment was carried out using Shimadzu HPLC LC2010 which consisted of a column RP - C18 5μ (250×4.6 mm) LiChrospher® 100, Isocratic pump mode with the flow rate of 1 ml/minute, UV Detector wavelength at 248 nm and the injection volume is 10 μL. The mobile phase consisting of buffer pH 6.7 and acetonitrile in the ratio of 72:28. The column oven temperature and Sample cooler temperature was maintained at 35oC and 6oC respectively. All solutions were degassed by ultrasound. Mobile phase was filtered through 0.45 μm nylon filter.

Standard and sample solutions

**Standard solutions:** Accurately weighed about 50 mg of Tigecycline working standard and transferred into a 100 ml clean, dry volumetric flask, about 5 ml of diluent A (water) is added and sonicated to dissolve. The volume is diluted with diluent B (dissolved about 4.35 g of Di-potassium hydrogen phosphate and 0.5 g of sodium hydrogen sulphite in 1000 ml of water, and adjusted the pH to 8.00 (±0.05) with dilute potassium hydroxide solution and mixed well) and mixed well to get a concentration of 500 μg/ml (stock I). Aliquots of 1ml, 2ml, 3 ml, 4 ml and 5 ml of standard stock solution is transferred into a 50 ml volumetric flask and diluted the volume with diluent B and mixed well to get a concentration range between 10 to 50 μg/ml. The Internal standard solution, oxytetracycline was prepared in the same manner as tigecycline standard solution.

**Mobile phase:** prepared a degassed mixture of buffer pH 6.7 (About 5.75 g of ammonium dihydrogen phosphate is dissolved in 1000 ml water, 30 ml of triethylamine is added and adjusted the pH to 6.7 (±0.05) with orthophosphoric acid solution. To this 0.5 g of EDTA disodium dihydrate is added and mixed well) and acetonitrile in the ratio of 72:28. Filtered the solution through 0.45 μm membrane filter.

**Sample preparation:** Reconstituted two sample vials each with 10 ml of diluent A. Pooled both vials and took 10 ml pooled sample into a 100 ml volumetric flask, 50 ml diluent B was added, sonicated for 10 min and diluted to volume with diluent B and mixed well. 1 ml of sample stock solution was transferred to 50 ml volumetric flask and diluted with diluent B and mixed well. Sample solution was filtered through a 0.45 μ syringe filter before injection.

Results and Discussion

Method Development and Optimization

Manipulation of mobile phase composition is a key factor for optimizing the separation and elution of the analytes in HPLC. Taking into consideration, different parameters like resolution, peak shape etc, and best results were obtained by addition of EDTA disodium dihydrate and by the addition of orthophosphoric acid for pH adjustment. In order to increase the sensitivity of the method, detection wavelength was selected as 248 nm at which tigecycline shows maximum absorption. The retention times for tigecycline and oxytetracycline were 10.6 and 3.6 respectively. Total running time for one sample was found to be 15 min.

**Method Validation:** Validation of the proposed method was performed with respect to specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness according to ICH guidelines[22, 23].

Specificity

Specificity is described as the ability of a method to discriminate the analyte from all potential interfering substances. It was evaluated by injecting analytical placebo (containing all ingredients of the formulation except the analyte) which is prepared according to the sample preparation procedure. To identify the interference by these excipients, a mixture of the inactive ingredients (placebo) was spiked with standard and internal standard substances. The representative chromatograms showed no other peaks, which confirm the specificity of the method.

The specificity of the proposed method demonstrated that the excipients from sample and diluents do not interfere in the drug peak. The chromatogram is presented in Fig.2.

Linearity

The various concentrations of working standard solutions of Tigecycline were made by pipetting aliquots...
of 2ml, 4 ml, 6 ml, 8 ml, 10 ml from stock-II separately into series of 10 ml volumetric flask and diluted to 10 ml to get the final concentration of 10 to 50 μg/ml solutions. About 20 μl of each of these working standard solutions of tigecycline ranging from 10 to 50 μg/ml were injected into chromatograph at a flow rate of 1 ml/min. Retention time and peak area obtained were recorded and standard curve was plotted (Fig.1) and linearity curve was defined by the following equation: \( y = 43931x + 62423 \), \( r = 0.9967 \), where \( y \) is the area and \( x \) is the concentration expressed in μg/ml (\( n = 3 \)). The equation of linear regression and statistical data for tigecycline are presented in Table 1. The linearity of the calibration curve for the drug was validated by the high value of the correlation coefficient.

**LOD and LOQ:** The limit of detection is defined as \( \text{LOD} = 3.3 \sigma / s \), where \( \sigma \) denotes standard deviation of response and \( s \) denotes slope. The limits of detection was determined as 0.052 μg/ml.

The limit of quantification is defined as \( \text{LOQ} = 10 \sigma / s \). The limits of quantification is determined as 0.157 μg/ml.

For the estimation of limit of detection (LOD) and limit of quantification (LOQ), visualization method was followed. In visualization method, lower dilutions of the standard drug of tigecycline was successively prepared, injected into the chromatograph and response obtained was recorded. Results are presented in Table 1.

**Precision:** The method was investigated with respect to system suitability test, method precision and intermediate precision. System suitability test ensures that the analytical system is working properly and can give accurate and precise results. Results are depicted in Table 3. System suitability test and method precision was carried out to monitor repeatability and reproducibility. Method precision was assessed by injecting successive six injections of standard solution (six replicates) into a HPLC chromatograph, the peak area and chromatograms obtained recorded. The percentage relative standard deviation was calculated for peak areas of replicates. The results are shown in Table 2.

**Table 2**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tigecycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± standard error</td>
<td>1197042 ± 283.35</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>693.927</td>
</tr>
<tr>
<td>Relative standard deviation (%)</td>
<td>0.058</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>System suitability factor</th>
<th>Tigecycline</th>
<th>acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>26439.78</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>HETP (mm)</td>
<td>37.82</td>
<td>–</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>0.903</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Resolution</td>
<td>14.89</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

Intermediate precision (ruggedness) was determined by the assay of sample sets in three independent series in the same day (intra-day precision) and three consecutive days (inter-day precision), within each series every sample was injected three times. The peak area and the chromatograms were recorded. The percentage assay of drug was calculated from the peak areas of replicates. The RSD values of intra-day and inter-day varied from 0.049 to 1.68 % (Table 4) showing that the intermediate precision of the method was satisfactory.
Accuracy

The accuracy of the proposed method was tested by recovery experiments. Recovery experiments were performed by taking sample concentrations and spiking with analyte concentrations at five different levels (10 μg/ml, 20 μg/ml, 30 μg/ml, 40 μg/ml, 50 μg/ml). Recovery efficiency of tigecycline from sample was sufficient, greater than 101.2% (Table 5).

<table>
<thead>
<tr>
<th>Amount of sample*</th>
<th>Amount of Std added*</th>
<th>Amount founda</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>10</td>
<td>41.95 ± 0.03</td>
<td>102.5</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>52.35 ± 0.04</td>
<td>104.7</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>52.35 ± 0.04</td>
<td>102.5</td>
</tr>
<tr>
<td>30</td>
<td>40</td>
<td>61.52 ± 0.07</td>
<td>101.2</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>82.85 ± 0.06</td>
<td>103.5</td>
</tr>
</tbody>
</table>

*aMean ± standard deviation (μg/ml),
*bconcentration - (μg/ml)

Robustness

Robustness relates to the capacity of the method to remain unaffected by small but deliberate variations in the flow rate and mobile phase ratio. The recoveries obtained for tigecycline were not different compared to the actual contents. Results are presented in Table 6.

<table>
<thead>
<tr>
<th>Change in % Assay</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (ml)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>98.96</td>
</tr>
<tr>
<td>1.2</td>
<td>95.82</td>
</tr>
<tr>
<td>Mobile phase ratio (Buffer:acetonitrile)</td>
<td></td>
</tr>
<tr>
<td>(80:20)</td>
<td>97.4</td>
</tr>
<tr>
<td>(72:28)</td>
<td>98.96</td>
</tr>
</tbody>
</table>

Assay

The proposed method was also evaluated by the assay of tigecycline in nanoparticle formulation. The results are mentioned in Table 7.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>mg/10 ml suspension</th>
<th>Found ± SD (mg)</th>
<th>% assay</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tigecycline</td>
<td>50</td>
<td>50.99 ± 0.026</td>
<td>101.9</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Conclusion

This paper describes a sensitive HPLC method of analysis of tigecycline in bulk and nanoparticle based suspension. The method has linearity and recovery within the range of standard curve. The method precision (repeatability), intra- and intra-day precision and accuracy of the assay is less than 2 %, which certify precise measurements of tigecycline in different prevailing conditions. The method is advantageous as it, does not use complicated instrumentation like MS/MS detection systems and show low cost conditions. Sample preparation procedure is simple with a short chromatographic run time making the method appropriate for processing multiple samples in a limited period of time. Hence it can be concluded that, the method is simple, quick, specific and reproducible, and has been successfully practical to monitor tigecycline in bulk and nanoparticle based suspension.

REFERENCES