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Rapid turbidimetric assay to potency evaluation of tigecycline in lyophilized powder

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ABSTRACT

Tigecycline, a first-in-class glycylcycline and an analog of the semisynthetic antibiotic minocycline, is a potent, broad-spectrum antibiotic that acts by the inhibition of protein translation in bacteria. This glycylcycline inhibits Gram-positive, Gram-negative, atypical, anaerobic and antibiotic-resistant organisms. There is no microbiological analytical method for tigecycline in lyophilized powder reported yet. Thus, this paper reports the development and validation of a simple, sensitive, accurate and reproducible turbidimetric method to quantify tigecycline in lyophilized powder, using *Staphylococcus aureus* as microorganism test and 3 × 3 parallel line assay design, with twenty tubes for each assay. The validated method showed good results of linearity in the concentration range from 3 to 4.32 μg/mL ($r^2 = 0.9999$), selectivity, precision, robustness and accuracy of 99.74%. The results demonstrated the validity of the proposed bioassay, which allows reliable quantitation of tigecycline in pharmaceutical samples and therefore can be used as a useful alternative methodology for the routine quality control of this medicine.

1. Introduction

Glycylcyclines, discovered in 1993, are structural analogues of tetracycline designed to avoid resistance mediated by efflux and ribosomal protection (Chopra, 2001). Tigecycline, a novel, first-in-class glycylcycline, is a potent, broad-spectrum antibiotic that acts by the inhibition of protein translation in bacteria (Hoffmann et al., 2007). Tigecycline is structurally derived from minocycline by adding a tert-butylglycylamido side chain to carbon 9 of the D ring of the tetracycline backbone (Pankey, 2005). Chemically, tigecycline is [(4S,4aS,5aR,12aS)-9-(2-tert-butylaminoacetylamino)-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 Da (Hoffmann et al., 2007; Doan et al., 2006).

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 (Bauer et al., 2004). This antibiotic has been shown to be as effective and safe as standard antimicrobial therapy for the treatment of adults with complicated intra-abdominal infections, complicated skin and skin structure infections, and community acquired bacterial pneumonia (Stein and Babinchak, 2013). It has also been evaluated as monotherapy for other serious infections in human clinical trials as a result of its microbiological, pharmacodynamic and pharmacokinetic properties (Chopra, 2001; Zhanel et al., 2004).

There currently exists a disturbing global trend that suggests the coming of a new post-antibiotic era, in which there are few antimicrobials available to treat new and emerging pathogens, fuelled by the use, overuse, and misuse of antibiotic therapy (Peterson, 2008). There is a need to develop new agents that overcome existing mechanisms of resistance displayed by multidrug-resistant bacteria (Bhattacharya et al., 2009). In this context, the development of new antimicrobials with activity against resistant pathogens and the study of analytical methodology to assure its quality, represent important clinical practice advance.

There are few methods described to analyze tigecycline and its metabolites, using techniques such as spectrophotometry (Silva et al., 2012) and HPLC-UV and HPLC/MS/MS (Li et al., 2004; Bradford et al., 2005; Muralidharan et al., 2005; Conte Jr et al., 2005; Ji et al., 2007, 2008; Hoffmann et al., 2007; Silva and Salgado, 2012; D'Avolio et al., 2013; Ozcimen et al., 2014; Xie et al., 2014). It is already known that liquid chromatography methods are more accurate, precise and specific than microbiological assays (Ródenas et al., 1995), however, the low cost and simple procedures of the bioassays have allowed them to become an alternative methodology for the drug potency assessment in pharmaceutical formulations, in addition, this assay can reveal subtle changes not demonstrable by conventional chemical methods (The United States Pharmacopoeia, 2011). Physicochemical methods used to quantify antimicrobial agents, although accurate, are not able to

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indicate the true biological activity of the drug. For this reason, microbiological methods are used to determine the potency of antimicrobial agents and they play an essential role in the manufacturing processes and quality control of these drugs (Moreno and Salgado, 2007; Vieira et al., 2012). Thus, it was considered important to develop an alternative method for tigecycline determination for application in routine quality control of these pharmaceutical dosage forms.

Additionally, the possibility to use alternative analytical methods fully validated for antibiotics, such as turbidimetric microbiological assay, which is a simple and operationally inexpensive one, represents a great advantage for quality control laboratories that do not have specialized and sophisticated instruments (Souza et al., 2006; Schmidt et al., 2009). Standard plate diffusion assays for antibiotics, although adequate, often do not provide the rapid and accurate assay information for the large number of test samples generated by development and control needs. Excellent precision can be achieved with a manual turbidimetric microbiological assay provided special care is taken in all of the operational details of the assay (Pitkin et al., 1974; Vieira et al., 2014).

Studies with tigecycline for microbiological testing showed that this antimicrobial agent should be incorporated in the culture medium on the day of use, or when conducted in liquid media, it must be freshly prepared and degassed in order to reduce the amount of oxygen in the medium. An alternative to fresh medium prepared is the addition of oxirase enzyme to the culture medium, which also decreases the concentration of oxygen present (Hope et al., 2005). It was observed that the activity of tigecycline against diverse population of bacteria was higher when compared to fresh media with different culture media preparation times (Petersen and Bradford, 2005).

Tigecycline is commercially available, but at the moment, there is no microbiological method for the analysis of this drug described in the literature. Considering that the turbidimetric assay has the advantage of reduced analysis time when compared to the agar diffusion method, where the analysis time is 24 h, the aim of this work was to propose a rapid turbidimetric method for the analysis of tigecycline in lyophilized powder, contributing to improve the quality control and assuring the therapeutic efficacy.

2. Experimental

2.1. Chemicals

The tigecycline reference substance was purchased from Sequoia Research Products (Oxford, UK). The batches of Tygacil® (Wyeth, USA) lyophilized powder, containing 50 mg of tigecycline were obtained from commercial sources within their shelf-life period. The samples contain lactose as the unique excipient. The culture media tryptic soy broth (TSB) and tryptic soy agar (Acumedia Manufacturers, Mi, EUA) were used for the method. Analytical grade formaldehyde (Qhemis, SP, Brazil) was used to interrupt the growth of microorganisms.

2.2. Apparatus

Incubation of microorganisms was performed using a Shaker incubator MA420 model (Marconi, SP, Brazil). A photometer Q-798DRM (Quimis, SP, Brazil) was used to determine the culture absorbance. The software Microsoft Excel (2007) was used to construct the calibration curves. The RP-LC method was performed on a Waters LC system (Waters Corporation, Milford, Massachusetts, USA) equipped with a Waters® 1525 binary pump, a Rheodyne Breeze 7725i manual injector and a Waters® 2487 UV detector. The peak areas were integrated automatically by computer using an Empower 2 software program. The chromatographic separation was carried out on a reversed-phase Phenomenex (Torrance, USA) Luna C_{18} column (250 mm × 4.6 mm I.D.).

2.3. Preparation of reference substance solution

The stock solution was prepared by weighing accurately, 12.50 mg of tigecycline reference substance, transferred to 100 mL volumetric flask and diluted to volume with ultrapure water, obtaining a concentration of 125 μg/mL of tigecycline. This solution was diluted with ultrapure water to a concentration of 25 μg/mL. Aliquots of 3.0, 3.6 and 4.32 mL of this solution were transferred to 25 mL volumetric flasks, the volumes of which were completed with ultrapure water in order to obtain working solutions with concentrations of 3.0, 3.6 and 4.32 μg/mL, respectively.

2.4. Preparation of sample solutions

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 ultrapure water to concentration of 125 μg/mL of the active pharmaceutical ingredient. After, the same reference substance dilutions were carried out.

2.5. Preparation of culture medium

The medium was prepared as indicated in their respective label being dissolved in water under heating, distributed in test tubes (10 mL/tube) and autoclaved (conditions: 121 °C, 1 atm) for 15 min. After autoclaving, the tubes were cooled and immediately used in the bioassay.

2.6. Turbidimetric assay

For the preparation and standardization of inocula, the strain *Staphylococcus aureus* was inoculated, with a platinum loop, into tryptic soy broth and incubated at 35 °C ± 2 °C, for 23 h before the assay, for the growth of *S. aureus*. The bacteria, previously incubated in tryptic soy broth, were diluted with pure tryptic soy broth to achieve a suspension turbidity of 25% ± 2% (transmittance), using a photometer with a wavelength of 580 nm and a 10 mm absorption cell, against tryptic soy broth as blank. The bioassay was performed using the 3 × 3 parallel line assay design (three doses of the standard and three doses of the sample). 800 μL of the standardized *S. aureus* suspension was added to twenty test tubes containing 10 mL of tryptic soy broth. In nine of these tubes, 200 μL of standard working solutions was added (at the concentrations of 3.0 (S1), 3.6 (S2) and 4.32 (S3) μg/mL, respectively), and each concentration was performed in triplicate. In the other nine tubes, the same was carried out with the working sample solutions (T1, T2 and T3). After that, the test tubes were incubated with shaking at a temperature of 35.0° ± 2.0 °C for 3 h. After the incubation period, the microbial growth was interrupted by the addition of 0.5 mL of 12% formaldehyde solution to each tube. Then, the photometer was reset by the test tube containing a negative control (10 mL of tryptic soy broth containing 0.5 mL of the formaldehyde solution) and the absorbance values were determined for each tube at a wavelength of 530 nm. As a positive control of the test, one tube containing 10 mL of tryptic soy broth, 800 μL of the standardized microorganism suspension and, after incubation, 0.5 mL of the formaldehyde solution was performed.

2.7. Calculation of activity and method validation

To calculate the activity of tigecycline, the Hewitt equation was used (Hewitt, 2003). The assays were calculated statistically by the linear parallel model and regression analysis and verified using analysis of variance (ANOVA). The method was validated using samples of pharmaceutical formulations with the label claim of 50 mg by determinations of the following parameters: specificity, linearity, precision, accuracy and robustness following the International Conference on Harmonisation (ICH) guidelines (ICH, 2005).