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Rational development and validation of a new microbiological assay for linezolid and its measurement uncertainty

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ABSTRACT

The aim of this work was to develop and validate a new microbiological assay to determine potency of linezolid in injectable solution. 2^4 factorial and central composite designs were used to optimize the microbiological assay conditions. In addition, we estimated the measurement uncertainty based on residual error of analysis of variance of inhibition zone diameters. Optimized conditions employed 4 mL of antibiotic 1 medium inoculated with 1% of *Staphylococcus aureus* suspension, and linezolid in concentrations from 25 to 100 μg mL^{-1}. The method was specific, linear ($Y=10.03X+5.00$ and $Y=9.20X+6.53$, $r^2=0.9950$ and 0.9987, for standard and sample curves, respectively), accurate (mean recovery=102.7%), precise (repeatability=2.0% and intermediate precision=1.9%) and robust. Microbiological assay's overall uncertainty (3.1%) was comparable to those obtained for other microbiological assays (1.7–7.1%) and for determination of linezolid by spectrophotometry (2.1%) and reverse-phase ultra-performance liquid chromatography (RP-UPLC) (2.5%). Therefore, it is an acceptable alternative method for the routine quality control of linezolid in injectable solution.

1. Introduction

Antimicrobial agents are one of the most used substance classes worldwide. They are used as preservatives in foods, medicines and industrial products, in hospital and industrial disinfection of surfaces, instruments and tissues and in the treatment of infectious diseases. The effectiveness of antimicrobial agents depends on the identity, purity and their activity against the specific microorganism. The clinical use of antimicrobial agents is an issue, particularly due to the emergence of resistant strains [1–3]. On the other hand, only a few new molecules have been developed and approved for clinical use in recent decades. Most of these new antibiotics belong to known classes, such as β-lactam and quinolone, and just one of these belongs to a new class, oxazolidinone [1–3].

Linezolid belongs to a new class of antibiotics, oxazolidinones, and it was approved for clinical use in 2000. The mechanism of action of oxazolidinones appears to be unique in that it blocks the initiation of protein synthesis, not of the latter steps. Chemically, linezolid is an (S)-N-[[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] acetamide (Fig. 1). It is used to treat serious infections caused by Gram-positive bacteria resistant to several antibiotics, including vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) [1–3].

The methods reported in the literature for determination of linezolid include ultraviolet (UV) spectrophotometry, high performance liquid chromatography (HPLC) UV-detection and fluorescence detection, capillary electrophoresis and thin-layer chromatography (TLC) followed by densitometric analysis and microbiological assay [4–11]. Despite that these methods present good reproducibility and are widely used in pharmaceutical quality control laboratories, they do not allow for evaluating antimicrobial activity (potency) [12–14].

The potency of antimicrobial agents may be evaluated according to their ability to inhibit microbial growth in appropriate conditions. In agar diffusion microbiological assay, two phenomena occur simultaneously: 1) diffusion of antimicrobial agent; and 2) microbial growth. According to Fick's law of diffusion, antimicrobial concentration can be estimated as a function of the initial concentration (at the well, cylinder or paper disc), a diffusion constant, time and the square of diffusion distance. Simultaneously, microbial growth occurs as a function of the initial burden load (inoculum amount), lag phase time, generation time and time of incubation [15–19]. A reduction in the antimicrobial activity can reveal subtle alterations that cannot be demonstrated through chemical methods. In addition to this, biological assays do not require specialized equipment or high toxicity solvents [18–23].

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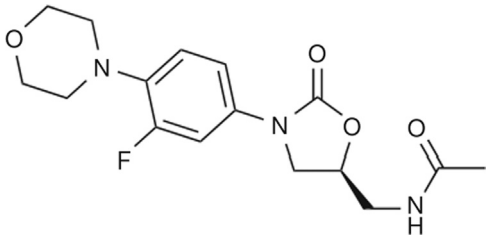

Fig. 1. Chemical structure of linezolid.

Traditionally, development of analytical methods has involved monitoring the influence of one factor at a time on the experimental results [24–26]. This approach may be tedious and slow; in addition, it does not include the interactive effects among the variables studied [24–26]. Factorial design and response surface methodology may be useful tools in the development of analytical methods, since they allow the evaluation of multiple factors in experimental results [24–26]. When a microbiological assay is performed, it is highly advisable to adopt an experimental design that, without further effort, delivers the best results and provides solid assay validity. The factorial design and response surface methodology may be useful in optimizing conditions which provide better results concerning the linearity, regression and parallelism of standard and sample curves [27,28].

Moreover, it is also important to assess the quality of the microbiological assay results. One useful measure of this is measurement uncertainty [29–34]. Measurement uncertainty provides additional information that may be useful for compliance or non-compliance decisions [34–39]. The uncertainty in the results may arise from many possible sources, including sampling, matrix effects and interferences, environmental conditions, uncertainties of mass and volumetric equipments, uncertainties of spectrophotometric and chromatographic equipments, uncertainties of biological and microbiological responses, purity of reagents and chemical reference substances, method validation and random variability [40–55].

The aim of this work is to develop and optimize a microbiological assay for linezolid, using factorial design and response surface methodology. This work also aims at validating the microbiological assay and evaluating its measurement uncertainty.

2. Materials and methods

2.1. Instruments

Calibrated volumetric flasks and pipettes, stainless steel cylinders, and a microbiological incubator (Nova Ética, Brazil) were used in the microbiological assay. Also, a calibrated inhibition zone reader (Haloes caliper, IUL) with accuracy of 0.1 mm was used in the microbiological assay. The experimental design and statistical analysis of the data were performed using a Minitab™ 16 software.

2.2. Reagents and reference materials

Antibiotic 1 culture medium (Anti 1, beef extract 1.5 g/L, yeast extract 3.0 g/L, pancreatic digest of casein 4.0 g/L, peptone 6.0 g/L, dextrose 1.0 g/L and agar 15.0 g/L) was obtained from AES laboratory (France). Tryptic soy agar culture medium (TSA, pancreatic digest of casein 15.0 g/L, papaic digest of soybean 5.0 g/L, sodium chloride 5.0 g/L and agar 15.0 g/L) was obtained from Difco/BD (USA). Sodium chloride was obtained from INLAB (Brazil). Linezolid working standard (Sigma-Aldrich, Lot 020M4707V) was characterized by assessment of its identification and purity using nuclear magnetic resonance (NMR), infrared spectroscopy (FTIR) and thermo-gravimetric analysis (TGA) in order to be used in the development and validation of the microbiological assay. Zyvox samples were obtained from Pfizer. Standard and sample solutions were diluted using a phosphate buffer pH 6.0 (potassium phosphate monobasic 8.0 g/L and potassium phosphate dibasic 2.0 gL). *S. aureus* (ATCC 6538) and *Kocuria rhizophila* (ATCC 9341) were obtained from the Instituto Adolfo Lutz (São Paulo, Brazil).

2.3. Development and optimization of microbiological assay

A 2^4 factorial design was used to verify the most important parameters among choice microorganism-test, choice of culture media, seeded layer volume, inoculum amount and concentration of linezolid. The conditions of 2^4 factorial experiments are described in Table 1. Microorganism-test and culture medium were chosen based on the factorial design.

Then, a central composite design (CCD – surface response methodology) was used to optimize the volume of seeded culture medium, inoculum amount and concentration of linezolid employed for microbiological assay. The conditions of CCD are listed in Table 2. Optimized conditions were established based on estimated inhibition zone sizes that provide good regression, linearity and parallelism of standard and sample curves.

2.4. Validation and uncertainty of microbiological assay

The method we developed and optimized was validated by assessing specificity/selectivity, linearity, precision (repeatability and intermediate precisions) and accuracy. Standard and sample curves were also tested for regression, lack of deviation and lack of parallelism. Robustness was evaluated using the central composite design during development and the optimization microbiological assay method. The influence of the volume of seeded culture medium and inoculum used for microbiological assay was evaluated simultaneously. In addition, the measurement uncertainty of linezolid potency was estimated, based on the residual error of analysis of variance for inhibition zones diameters of standards and samples.

Table 1
A 2^4 factorial design to establish the most significant factors affecting the inhibition zone diameters of linezolid microbiological assay.

Microorganisms	Culture media	Volume of seeded culture medium[a] (mL)	Inoculum proportion[b] (%)
Staphylococcus aureus	TSA	4.0	1.0
Staphylococcus aureus	TSA	4.0	2.0
Staphylococcus aureus	TSA	5.0	1.0
Staphylococcus aureus	TSA	5.0	2.0
Staphylococcus aureus	Anti 1	4.0	1.0
Staphylococcus aureus	Anti 1	4.0	2.0
Staphylococcus aureus	Anti 1	5.0	1.0
Staphylococcus aureus	Anti 1	5.0	2.0
Kocuria rhizophila	TSA	4.0	1.0
Kocuria rhizophila	TSA	4.0	2.0
Kocuria rhizophila	TSA	5.0	1.0
Kocuria rhizophila	TSA	5.0	2.0
Kocuria rhizophila	Anti 1	4.0	1.0
Kocuria rhizophila	Anti 1	4.0	2.0
Kocuria rhizophila	Anti 1	5.0	1.0
Kocuria rhizophila	Anti 1	5.0	2.0

[a] Aliquots of 21 mL of the Anti 1 or TSA are employed as base layer.
[b] Suspension prepared in 0.9% sodium chloride solution and diluted to obtain $25 \pm 3\%$ of transmittance at 580 nm. Linezolid concentrations of 25, 50 and 100 μg $mL^{-1}$are used.