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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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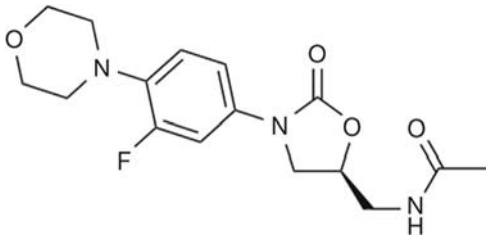

Fig. 1. Chemical structure of linezolid.

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.

Table 2
Response surface methodology used to optimize the microbiological assay for linezolid with respect to volume of culture medium and proportion of inoculum, considering *Staphylococcus aureus* and antibiotic 1 as microorganism and culture medium, respectively.

Volume of seeded culture medium[a] (mL)	Inoculum proportion[b] (%)	Antibiotic concentration ($\mu g\ mL^{-1}$)
4.0	1.0	25, 50 and 100
4.0	2.0	25, 50 and 100
5.0	1.0	25, 50 and 100
5.0	2.0	25, 50 and 100
4.5	1.5	25, 50 and 100
4.5	1.5	25, 50 and 100
4.5	1.5	25, 50 and 100
4.5	1.5	25, 50 and 100
4.5	1.5	25, 50 and 100

[a] Aliquots of 21 mL of the Anti 1 or TSA were 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.

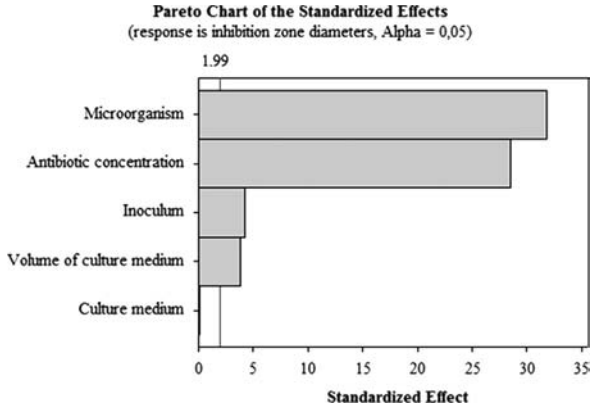

Fig. 2. Pareto chart shows influence of microorganism, culture medium, volume of seeded culture medium, inoculum and antibiotic concentration on the inhibition zone diameters of linezolid microbiological assay.

3. Results and discussion

3.1. Development and optimization of microbiological assay

The development and optimization of microbiological assay for linezolid were performed using the factorial design and response surface methodology. The 2^4 factorial design was performed (Table 1) to establish the most significant factors in the inhibition zone diameter. According to the Pareto chart results (Fig. 2), microorganism test and antibiotic concentration have a greater influence on the inhibition zone diameters of the linezolid microbiological assay. We must pay attention to the fact that the microorganisms showed a greater influence in the inhibition zone diameters than antibiotic concentration. In other words, the choice of the microorganism to be used in the microbiological assay is a critical issue.

In microbiological agar diffusion method for antibiotics, two phenomena occur simultaneously: a) the diffusion of antibiotic; and b) microbiological growth. The inhibition zone diameters depend on these two phenomena. The susceptibility of microorganism-test may significantly affect the inhibition zone diameters (Fig. 3 – Microorganism). *K. rhizophila* seems to be more susceptible to linezolid than *S. aureus*. However, delimitation of halos using *S. aureus* was better, because the growth was denser.

The culture medium composition may affect microbial growth and antibiotic diffusion, which may affect the inhibition zone diameters. Higher contents of agar, dextrose and salt may reduce the diffusion of the antibiotic, which will decrease the inhibition zone diameters. On the other hand, the amount of nutrients may reduce the lag phase period and/or generation time of microorganisms, which will decrease the inhibition zone diameters. Culture medium pH may also affect both microbial growth and antibiotic diffusion. The slight differences among the inhibition zone diameters using TSA and Anti 1 may be explained by their differences in composition and pH (Fig. 3 – Culture medium) [15]. *S. aureus* and antibiotic 1 (Anti 1) were adopted as microorganism and culture medium, respectively, based on 2^4 factorial design results.

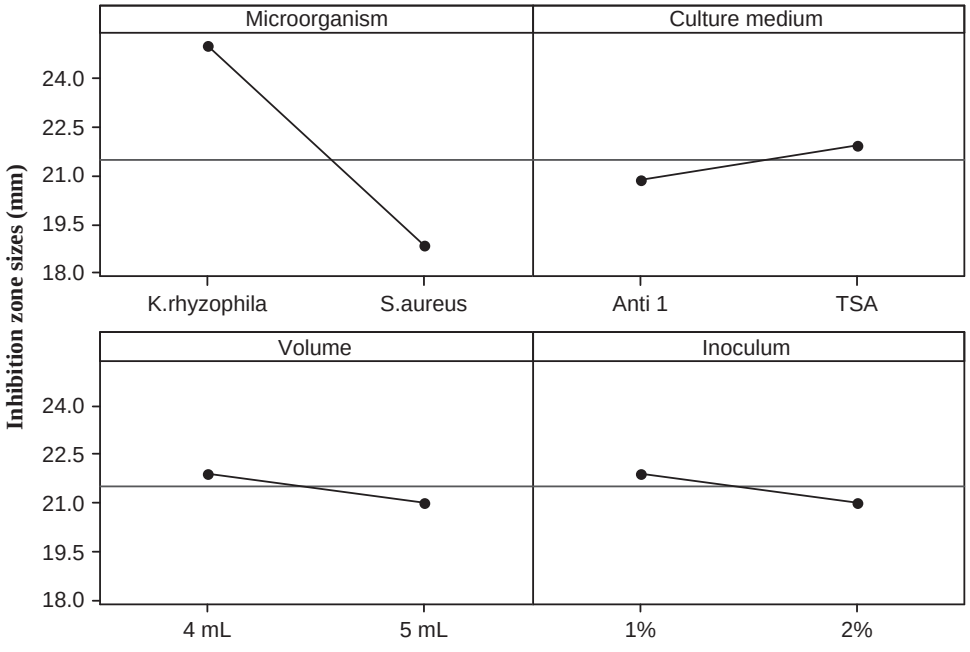

Fig. 3. Main effects plot to show the influence of microorganism, culture medium, volume of seeded culture medium and inoculum on the inhibition zone diameters of linezolid microbiological assay.

In addition to culture medium composition, the thickness of the seeded layer (which may be measured as the volume of seeded culture medium) is an important issue in the inhibition zone diameters. According to our results (Fig. 3 – Volume), the higher the volume of seeded culture medium, the smaller the inhibition zone diameters. These results are in accordance with the agar diffusion theory [15]. Double-layer (seed layer and base layer) plates are often used in microbiological assay, due to their advantages regarding monolayer plates (seed layer only). Base layer (prepared with about 20–21 mL) will provide nutritional demand of microbial growth that occurs in a very thin seeded layer (prepared with about 4–5 mL). The thinner the seed layer the bigger the inhibition zones, and consequently, the more sensitive the microbiological assay is. Moreover, the higher the microorganism inoculum the smaller the inhibition zone diameters, because a higher amount of antibiotic will be needed to inhibit the microbial growth. Our results are in accordance with these (Fig. 3 – Inoculum) [15]. Microbial suspension was prepared in 0.9% sodium chloride solution and diluted to obtain 25 ± 3% of transmittance at 580 nm to ensure the reproducibility of microorganism inoculum (about 10^6 CFU mL^{-1}).

A surface response methodology was used to optimize the microbiological assay for linezolid with respect to the volume of culture medium, proportion of inoculum and antibiotic concentration (Table 2). First, a full quadratic model was adopted to explain the inhibition zone diameters based on the volume of culture medium, proportion of inoculum and antibiotic concentration. Although the full quadratic model showed good adjustment ($p=0.985$ for lack-of-fit and $r^2=0.9221$), most of the quadratic factors and all of interactions were not significant. Therefore, an incomplete quadratic model is adjusted, as shown in Table 3. Also, three-dimensional plots of the response surface (inhibition zone diameters) as a function of volume of medium, proportion of inoculum and linezolid concentration are shown in Fig. 4. Response optimization analysis indicates the following experimental conditions: (a) *S. aureus* (ATCC 6538) as microorganism; (b) antibiotic medium 1 as culture medium; (c) double-layer plates prepared with 21.0 mL of base layer and 4.0 mL of seed layer; (d) seed layer inoculated with a proportion of 1.0% (v/v) of microbial suspension (25 ± 3% of transmittance at 580 nm); (e) incubation of plates at 37 ± 1 °C for 18–24 h; and (f) linezolid concentrations of 25, 50 and 100 μg mL^{-1}.

3.2. Validation and uncertainty of microbiological assay

Using the optimized conditions established, the method was validated by assessing specificity/selectivity, linearity, precision (repeatability and intermediate precision) and accuracy. There was no interference of excipients of linezolid pharmaceutical preparation, which indicates the specificity/selectivity of microbiological method. The microbiological assay showed good linearity in the range from 25 to 100 μg mL^{-1}, with linear equation of $Y=10.03X+5.00$ and $Y=9.20X+6.53$ and regression coefficient (r^2) of 0.9950 and 0.9987, for standard and sample curves, respectively (Fig. 5). The results of linearity indicate parallelism among standard and sample curves, which is important to confirm validity of potency results. In addition, the microbiological method was precise (repeatability = 2.0% and intermediate precision = 1.9%) and accurate (mean recovery = 102.7%). In addition, the central composite design allowed us to have a better understanding of the influence of the volume of seeded culture medium and inoculum amount on microbiological assay results. According to our results, inoculum amount affects the inhibition zone sizes more than the volume of seeded culture medium, as shown in Figs. 2–4. However, the microbiological assay may be considered robust to slight changes in these conditions.

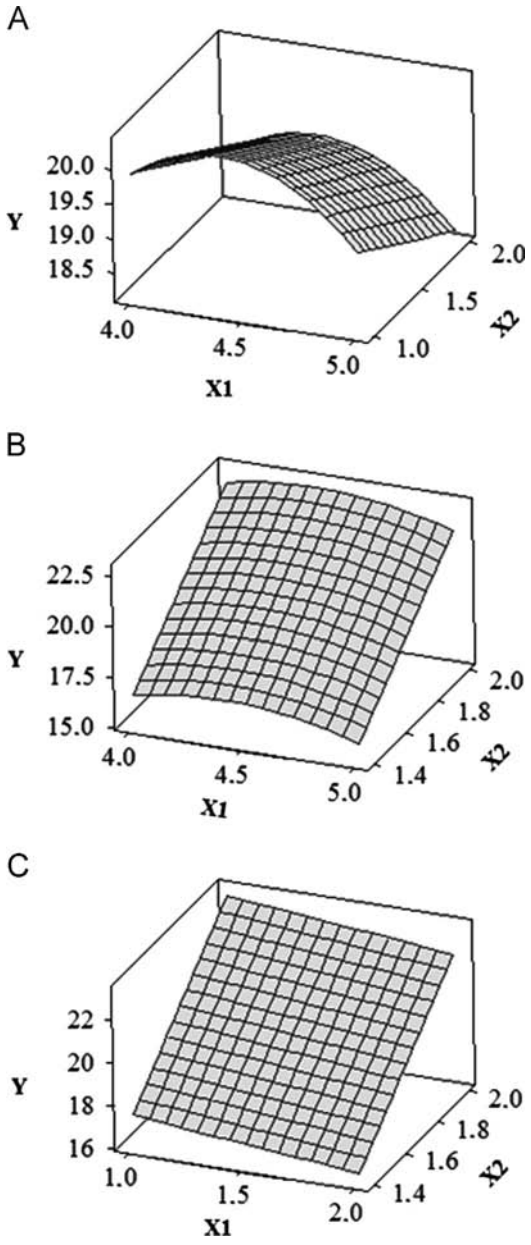

Fig. 4. Three-dimensional plots of the response surface for *Y* (inhibition zone diameters). (A) Variation of response *Y* as a function of *X*1 (volume of seeded culture medium) and *X*2 (proportion of inoculum). (B) Variation of the response *Y* as a function of *X*1 (volume of seeded culture medium) and *X*2 (linezolid concentration). (C) Variation of the response *Y* as a function of *X*1 (proportion of inoculum) and *X*2 (linezolid concentration).

Table 3
An incomplete quadratic model to explain the inhibition zone diameters based on volume of seeded culture medium, proportion of inoculum and antibiotic concentration.

Factors	Model	Coefficients
Constant	Linear	−51.1987
Antibiotic concentration	Linear	9.61206
Proportion of inoculum	Linear	−1.06944
Volume of medium	Linear	25.6250
Volume of medium	Quadratic	−2.91667

This model showed good adjustment ($p=0.993$ for lack-of-fit and $r^2=0.9198$).

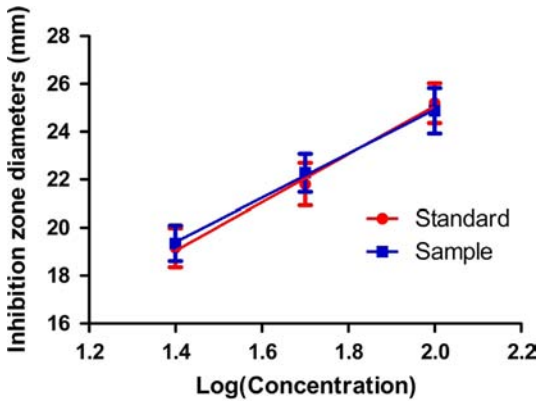

Fig. 5. Standard and sample curves of linezolid for microbiological assay using *Staphylococcus aureus* (ATCC 6538), antibiotic 1 culture medium, seeded-layer volume of 4 mL and 1% of inoculum proportion.

Most of uncertainty in a microbiological assay is associated with variability of inhibition zone diameters (within and between plates). Lourenço [52] and Ghisleni et al. [21] describe procedures to estimate the uncertainty of microbiological assay using analysis of variance (ANOVA), using different experimental designs. Also, uncertainty of microbiological assay may be estimated using method validation data [48]. According to our results, linezolid potency and its final uncertainty were found to be $102.7 \pm 3.1\%$. This uncertainty was comparable to those obtained for apramycin (6.9–7.1%) [52], cefazolin (6.2–6.7%) [54], caspofungin (1.7%) [21], and vancomycin (4.3%) [48]. This uncertainty was also comparable to those obtained from determination of linezolid by UV spectrophotometry (2.1%) [4], and RP-UPLC (2.5%) (our results not published yet).

The higher variability of microbiological assay may be explained because of the complexity of diffusion of antimicrobial agent and microbial growth. Several factors such as thickness of seeded layer (among plates and lack of uniformity within plates), amount of microorganism, composition of culture medium, variability of temperature and time of incubation, diffusion issues, and others may affect the inhibition zone diameters, and consequently, they will affect the measurement uncertainty of microbiological assay [15]. However, further studies should be done to determine the individual contribution of these factors to microbiological assay's overall uncertainty.

4. Conclusion

A new microbiological assay for quantification of linezolid was developed and optimized. The optimized conditions employed 4 mL of antibiotic 1 medium inoculated with 1% of *S. aureus* suspension, and linezolid in concentrations from 25 to 100 $\mu g\ mL^{-1}$. The method was specific, linear, accurate, precise and robust. The microbiological assay's overall uncertainty was comparable to those obtained for other microbiological assays and for determination of linezolid by spectrophotometry and RP-UPLC. Therefore, it is an acceptable alternative method for the routine quality control of linezolid in injectable solution.

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