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Development and validation of a stability-indicative agar diffusion assay to determine the potency of linezolid in tablets in the presence of photodegradation products

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ABSTRACT

Linezolid (LNZ) is one of the first commercially available (and most widely used) oxazolidinone antibiotics. This study describes the development and validation of a microbiological assay, applying the cylinder-plate method, for the determination of the antibiotic linezolid, as well as the evaluation of the ability of the method in determining the stability of linezolid in tablets. The validation method yielded good results and included linearity, precision, accuracy, robustness and selectivity. The assay is based on the inhibitory effect of LNZ upon the strain of *Bacillus subtilis* ATCC 9372 used as the test microorganism. The results of the assay were treated statistically by analysis of variance (ANOVA) and were found to be linear ($r^2 = 0.9998$) in the range of 20–80 $\mu g\,mL^{-1}$, precise (inter-assay: R.S.D. = 0.61) and accurate (R.S.D. = 1.7). The method developed and validated proved to be indicative of stability and capable of determining the decay of linezolid in the presence of photodegradation products. Comparison of bioassay and liquid chromatography by ANOVA showed no significant difference between methodologies. The results demonstrated the validity of the proposed bioassay, which is a simple and useful alternative methodology for LNZ determination in routine quality control.

1. Introduction

The oxazolidinones represent the first truly new class of antibacterial agents to reach the marketplace in several decades [1]. Linezolid (s)-N-[[3-[3-fluoro-4(4-morpholinyl) phenyl]-2-oxo-5-oxazolidinyl] methyl] acetamide (Fig. 1) was the first oxazolidinone to be developed and approved for clinical use. It is active against a range of bacteria, but its primary clinical role is the treatment of infections caused by aerobic Gram-positive organisms, including resistant strains such as vancomycin resistant enterococci, methicillin-resistant *Staphylococcus aureus* and penicillin-resistant pneumococci [2–5]. Assays reported in the literature for the determination of linezolid in biological fluids include HPLC using UV-detection [6–9], and fluorescence detection [10].

For the measurements in pharmaceutical dosage forms, the methods reported in literature are HPLC [11–13], capillary electrophoresis [14] and TLC followed by densitometric and first derivate spectrophotometry [15]. However, a microbiological assay for the determination of LNZ in raw material and tablets has not yet been reported. Microbiological assays are less accurate, precise and specific in comparison with LC assays [16]. However, the low cost and simple procedures of bioassays have allowed them to become an alternative methodology for drug potency assessment in pharmaceutical formulations [17–23]. This assay can reveal subtle changes not demonstrable by conventional chemical methods [24]. Bioassay is an ecological technique because it is not a residue or solvent producer. Moreover, microbiological assay requires no specialized equipment or toxic solvents. The microbiological assay is a method as sensitive as the chromatographic, and has no need to use organic solvents for their implementation. The aim of the present study was to develop and validate a simple, sensitive, precise, accurate and stability-indicative microbiological assay by agar diffusion using a cylinder-plate method to quantify LNZ in raw material and tablets as an alternative to the physicochemical methods described in the literature.

2. Experimental

2.1. Standard and reagents

LNZ reference substance was obtained from "Synfine Research" Chemical Company (Canada) and was analyzed by the HPLC and

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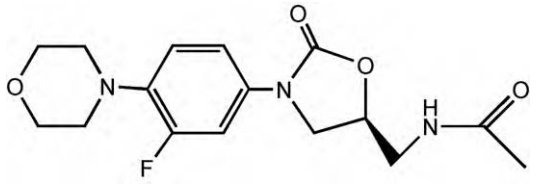

Fig. 1. Chemical structure of linezolid (CAS 165800-03-3).

was found to contain 99.3% of LNZ. The LNZ tablets, which were claimed to contain 600 mg of active drug, were obtained commercially. Each tablet (Zyvox®, Pfizer, Brazil) contains 600 mg of LNZ. Ultrapure water was obtained from a Milli-Q® Plus apparatus (Millipore®) and was used to prepare all solutions for the bioassay.

2.2. Preparation of standard solutions

Accurately weighed 10 mg LNZ reference standard were transferred to a 100 mL volumetric flask with 50 mL ultrapure water and shaken for 10 min (100 $\mu g\,mL^{-1}$). This was followed by making up to volume with water. Aliquots of 2, 4 and 8 mL this solution were diluted in potassium phosphate buffer pH 6.0 to give concentrations of 20, 40 and 80 $\mu g\,mL^{-1}$, which were used in the assay.

2.3. Preparation of sample solutions

An amount of powder equivalent to 100 mg LNZ was transferred to a 100-mL volumetric flask with ultrapure water and shaken for 20 min, followed by the dilution to volume with ultrapure water (1000 $\mu g\,mL^{-1}$). An aliquot of this solution (10 mL) was transferred to a 100 mL volumetric flask (100 $\mu g\,mL^{-1}$) and aliquots of 2, 4 and 8 mL were transferred to 10 mL volumetric flasks, completed with potassium phosphate buffer pH 6.0, to obtain the concentrations of 20, 40 and 80 $\mu g\,mL^{-1}$, respectively.

2.4. Microorganism and inoculum

The cultures of *Bacillus subtilis* ATCC 9372 were cultivated on Grove Randall number 1 agar (Merck) in the freezer and pealed to another Grove Randall number 1 agar (24 h before the assay) that was kept at 36 °C. The bacteria were suspended in tryptic soy broth (Difco) using a glass homogenizer. Diluted culture suspension of 25 ± 2% turbidity was obtained at 580 nm, using a suitable spectrophotometer and a 10-mm diameter test tube as an absorption cell against tryptic soy broth as blank. Portions of 1.0 mL of the inoculated tryptic soy broth were added to 50 mL of Grove Randall number 1 agar (Merck) at 47 ± 2 °C and used as the inoculated layer.

2.5. Agar diffusion bioassay

In order to assay the standard samples, 18 dishes were used, six each day. The agar was composed of two separate layers. The Grove Randall number 1 agar (20 mL) was poured into a 100 mm × 20 mm Petri dish as a base layer. After solidification portions of 5 mL of inoculated Grove Randall number 1 agar were poured onto the base layer. Six stainless steel cylinders of uniform size (8 mm × 6 mm × 10 mm) were placed on the surface of the inoculated medium. Three alternate cylinders were filled with 200 μL of the reference solutions and the other three were filled with the sample solutions. The bioassay plates were aerobically incubated at 35 °C for 18 h. The zone diameters (in mm) of the growth inhibition were carefully measured using a digital caliper (Starret) with a precision of at least 0.1 mm. Three assays a day were performed using 6 plates in each one. The sample results were compared with the standard curve obtained in the same experiment.

2.6. Calculation

To calculate the activity of LNZ in tablets the Hewitt equation was used. The assay was statistically analyzed by the linear parallel model and by means of regression analysis of variance [25].

2.7. Method validation

All experimental conditions of the proposed method were tested and adjusted prior to the validation in order to accurately determine the performance of the assay. The methodology was validated according to the International Conference on Harmonization and USP [26] by determination of the following operational characteristics: linearity, precision, accuracy, specificity and robustness. According to the Brazilian pharmacopoeia the limits of detection and quantification are not required for this category of assay [27].

2.7.1. Linearity

In order to assess the validity of the assay, three doses of the reference substance and three doses of the sample were used. The calculation of regression line by the method of least squares was employed.

2.7.2. Precision

Repeatability (intra-assay) and intermediate precision (inter-assay) were determined. Method repeatability was studied by analyzing samples of tablets, at the same concentration, within 1 day and under the same experimental conditions. The intermediate precision was evaluated by comparing the assays on 3 different days.

2.7.3. Accuracy

Accuracy was determined by adding known amounts of LNZ reference substance to the samples at the beginning of the process. Accurately weighed amounts of tablet equivalent of 100 mg LNZ were placed in three 100-mL volumetric flasks. 5, 10 and 15 mL of LNZ reference solution (100 $\mu g\,mL^{-1}$) were added. Potassium phosphate buffer pH 6.0 (50 mL) was added and the flasks were shaken for 20 min. This was followed by making up to volume with potassium phosphate buffer pH 6.0. The dilutions were made in potassium phosphate buffer, pH 6.0, to give final concentrations of 105.0, 110.0 and 115.0%, respectively. The solutions were transferred to the cylinders in the plate (see Section 2.5). The percentage recovery of LNZ reference added was calculated using the formula proposed by the AOAC.

2.7.4. Specificity

The ability of the proposed method to determine LNZ in the presence of the photodegradation products was assessed by comparing the results obtained in the bioassay with the degraded samples against the results of the HPLC methods described [13] for the same degraded samples. Tablet powder of LNZ was prepared and exposed to light to determine the effects of irradiation on the stability of LNZ in the solid state. Samples of LNZ exposure times of 0, 10, 30, 60 and 90 days were prepared and analyzed at a concentration of 20, 40 and 80 $\mu g\,mL^{-1}$.

2.7.5. Robustness

Robustness was determined by analyzing the same sample under a variety of conditions. The considered factors were incubation time, volume of the inoculated layer (thickness), and inoculum concentration. The variation (intradoses) of the mean diameter of the inhibition zones between the different assays was statistically analyzed by ANOVA.

Table 1
Conditions tested to establish the parameters for microbiological assay of LNZ.

Parameter	Condition
Standard curve ($\mu g\,mL^{-1}$)	20, 40 and 80; 5, 10 and 20
Buffer solution (pH)	6.0 and 8.0
Inoculum (%)	1.0 and 2.0
Microorganism	*Staphylococcus aureus* ATCC 6538 *Bacillus subtilis* ATCC 9372 *Staphylococcus epidermidis* ATCC 12228
Culture media	Grove Randall numbers 1 and 11 agar

2.7.6. Chromatographic conditions

An LC system consisting of Waters, model 1525 (Waters Chromatography systems, CA, USA) was connected to a UV/Visible waters 2487 and an injector fitted Rheodyne Breeze 7725i with a 20 μL loop. The chromatographic separation was carried out under isocratic reversed-phase conditions on a Symmetry Waters C18 column, 5 μm, 250 mm × 4.6 mm (Waters, USA). The mobile phase used was aqueous 1% acetic acid:methanol:acetonitrile (50:25:25, v/v/v). All analyses were done under isocratic conditions at a flow-rate of 1.0 mL min^{-1} and at room temperature.

2.7.7. Comparison of methods

The results obtained in this study were compared with those by a high-performance liquid chromatography (HPLC) method described previously.

3. Results and discussion

3.1. Analytical method

The development and validation of analytical methods for the potency determination of drugs has received considerable attention in recent years, mainly from regulatory agencies, because of their importance in pharmaceutical analysis. In this case, a microbiological assay was proposed as a suitable method for the determination of LNZ in tablets. The experimental conditions were adjusted to accurately determine the performance of the assay. Some parameters were tested earlier to establish the conditions described and shown in Table 1. A strain of *B. subtilis* ATCC 9372 was found to be an appropriate test microorganism allowing quantitation of LNZ. The potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a reference standard. During microorganism handling, all safety procedures, like the use of a mask, gloves, and safety glasses, were employed. All assays were performed in a laminar air flow cabinet, and the infected material was decontaminated before discarded. The absence of impurities in the reference standard used in the study is confirmed by a spectrum obtained by LC/MS/MS. According to the Brazilian pharmacopoeia the limits of detection and quantification are not required for this category of assay. For HPLC method the limit of detection and limit of quantification were 0.21 and 0.63 $\mu g\,mL^{-1}$.

3.2. Linearity

The calculation procedure usually assumes a direct relationship between the observed zone diameter and the logarithm of applied dose. The corresponding mean zone diameters for reference solutions were: 19.71 mm (R.S.D. = 0.63) for the lower dose (20 $\mu g\,mL^{-1}$), 19.57 mm (R.S.D. = 0.51) for the medium dose (40 $\mu g\,mL^{-1}$), and 22.60 mm (R.S.D. = 0.30) for the higher dose (80 $\mu g\,mL^{-1}$; Table 2). The calibration curves for LNZ were constructed by plotting log concentrations ($\mu g\,mL^{-1}$) versus zone diameter (mm) and showed good linearity between 20 and 80 $\mu g\,mL^{-1}$ range. The representative linear equation for LNZ was $y = 10.34 \ln x + 2.93$, where x is log dose and y is zone diameter. The coefficient of regression was $r^2 = 0.9998$. The experimental values obtained for the determination of LNZ in the samples are presented in Table 3. According to the Brazilian British and European Pharmacopoeias [27–29], if a parallel-line model is chosen, the two log dose–response lines of the preparations to be examined as well as the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as described in these official guidelines. There was no deviation from parallelism and linearity in the results obtained here ($P < 0.05$) (Table 4).

Table 2
Diameters of growth inhibition zones for LNZ standard solutions obtained for the standard curve.

Sample concentrations ($\mu g\,mL^{-1}$)	Diameters of growth inhibition zones[a] (mm)	Mean diameters of growth inhibition zones (mm)	R.S.D. (%)
20	16.42 16.25 16.44	16.37	0.63
40	19.69 19.54 19.50	19.57	0.51
80	22.68 22.56 22.56	22.60	0.30

[a] Each value is the mean of 6 plates.

Table 3
Between-day precision data for the bioassay of linezolid in samples of pharmaceutical formulations.

Sample in tablets (mg)	Day	Experimental amount[a] (mg)	Potency found (%)	Confidence limits ($P = 0.95$)	R.S.D. (%)
600	1	607.62	101.27	96.00–106.84	0.61
	2	612.14	102.14	98.34–106.10	
	3	614.88	102.48	98.34–106.80	

[a] Each value is the mean of 6 plates.

3.3. Precision and accuracy

The precision and accuracy of the assay were also demonstrated. The repeatability shows a mean R.S.D. of 0.61. The results obtained on different days (intermediate precision) show a relative standard deviation of 0.61 for tablets (Table 3). The accuracy is shown by the agreement between the accepted value and the value found to be 101.96 for tablets (Table 5).

Table 4
Statistical analysis of calibration graphs and analytical data in the determination of linezolid by bioassay.

Parameters	Microbiological assay
Regression analysis	
Slope (S.E.[a])	10.34 (0.16)
Intercept (S.E.[a])	2.93 (0.26)
Correlation coefficient (r^2)	0.9998
Analysis of variance	
Linear regression[b]	1903.98
Linearity deviation[b]	0.002

[a] Standard error of mean.
[b] Values in parentheses are the corresponding critical values for F at $P = 0.05$.

Table 5
Experimental values obtained in the recovery test for linezolid in tablets by microbiological assay.

Sample concentrations ($\mu g\,mL^{-1}$)	Concentration of added standard ($\mu g\,mL^{-1}$)	Concentration of found standard ($\mu g\,mL^{-1}$)	Percentage recovery[a]	Mean percentage recovery ± R.S.D.
20	0.5	0.49	98.6	
40	1.0	1.02	102.0	100.2 ± 1.70
80	1.5	1.5	100.0	

[a] Each value is the mean of 6 determinations.

Table 6
Conditions investigated in the robustness test.

Condition	Parameter	Stated potency (%)
Inoculated layer	4.5 mL	99.94
Incubation time	24 h	102.65
Inoculum concentration	2.2%	105.69

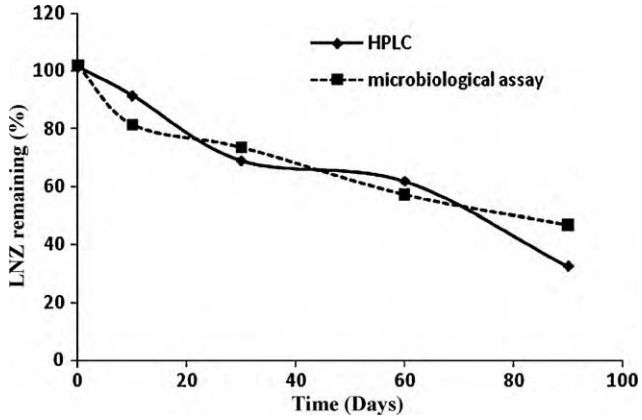

Fig. 2. Degradation profile of the LNZ commercial sample at the times 0, 10, 30, 60 and 90 days.

3.4. Robustness

Considering the high degree of variability of the bioassays and the drug assay purpose of the methodology, it was considered necessary to evaluate small variations in the analytical conditions. Therefore, in order to assess the robustness, some parameters were modified from the normal conditions: incubation time (24 h), volume (thickness) of the inoculated layer (5 mL), and inoculum concentration, as shown in Table 6.

3.5. Selectivity

To test the selectivity of the proposed method, tablet powder of LNZ was prepared and exposed to light to determine the effects of irradiation on the stability of LNZ in the solid state and was subsequently analyzed. The content of remaining LNZ in the sample was assessed by the pharmacopeial method and the proposed bioassay, at the 0, 10, 30, 60 and 90 days of the exposure time to the dry heat, resulting in the comparative degradation profiles of LNZ presented in Fig. 2. The LNZ has low photostability and when analyzed by both methods presented similar degradation profiles,

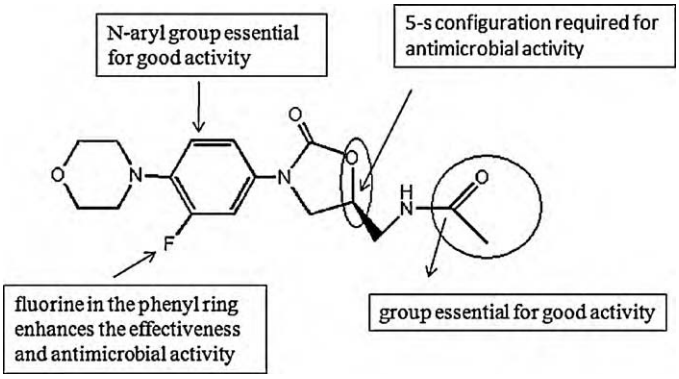

Fig. 3. Structure-activity of linezolid. Degradation of linezolid in positions circulated.

the ANOVA testing confirmed no significative difference between methods ($P > 0.05$).

The study showed that photodegradation of linezolid is likely under UVC light (254 nm) under the studied conditions. The analysis was performed by keeping the equivalent of three average weights of crushed tablets in a chamber with a mirror exposed to UVC light (254 nm), forcing extreme analysis. This study aimed to verify the decay of the levels of linezolid in tablets during 90 days and to ascertain what are the possible degradation products formed under this stress condition.

Samples exposed for 90 days in light-UVC were analyzed by microbiological assay. This method proves to be sensitive to the degradation of linezolid in UVC light, giving a decay characteristic of the content within 90 days (Fig. 2). In this case, it is assumed that the degraded organic groups are probably important for the antimicrobial activity of linezolid. According to the chemical structure of linezolid, it is possible to identify five groups that contribute to the antimicrobial activity (Fig. 3) [30]. With these data, it is assumed that the circulated groups were broken, reducing the antimicrobial activity (Fig. 3). The potency of linezolid tablets after 90 days of exposure was 46.81%.

These results show that the microbiological assay was specific and the impurities and degradation products did not interfere in the capacity of the method to assess the analyte.

3.6. Comparison of methods

The results obtained with the cylinder-plate assay were comparable with declared amounts and with those obtained by HPLC (Table 7). Analysis of variance indicated no significant differences

Table 7
Assay results of linezolid by 2 different methods.

Day	Liquid chromatography (%) (mean ± R.S.D.) ($n = 3$)	Mean[a] (%)	Microbiological[a] (%)	Mean[a] (%)
1	102.04 ± 1.77		101.27 ± 2.87	
2	99.45 ± 1.60	101.36 ± 1.65	102.14 ± 3.14	101.96 ± 0.61
3	102.60 ± 1.79		102.48 ± 1.40	

[a] Mean ± R.S.D (n = 3)

between these methods ($P < 0.05$). The quantification of antibiotic components by chemical methods such as HPLC, although precise, cannot provide a true indication of biological activity. Attempts to correlate antibiotic bioassay results with those from chemical methods have proved disappointing. Therefore, bioassays continue to play an essential role in manufacturing and quality control of antibiotic medicines, and yet still demand considerable skill and expertise to assure success. Although the biological assays have a high variability, the analysis of the obtained results demonstrated that the proposed method might be very useful for determination of this drug in pharmaceutical dosage forms.

4. Conclusions

The results indicated that the microbiological cylinder-plate assay demonstrated good linearity, precision, accuracy, robustness and stability-indication at concentrations ranging from 20 to 80 $\mu g\,mL^{-1}$, therefore, being an acceptable alternative method for the routine quality control of LNZ in raw materials and tablets. The method uses simple reagents, with minimum sample preparation procedures and generation of residues, encouraging its application in routine analysis.

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