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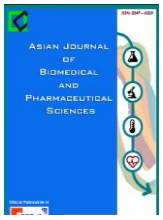

RESEARCH ARTICLE

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A Comparative Study of Linezolid Stability samples by Validated Spectrophotometric and Turbidimetic-Microbial Assay Methods

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Abstract

Linezolid is one of the first commercially available and most widely used oxazolidinone antibiotics. This study describes the development of a microbiological assay, applying the turbidimetric method for the determination of linezolid, as well as the evaluation of the ability of the method in determining the stability of linezolid against acidic, basic, neutral, thermal and photolytic degradants using *Staphylococcus aureus* ATCC 6538P as the test micro-organism. The results obtained with the microbial method were compared with the results obtained from the spectrophotometric method, developed and validated using ferric citrate and 1, 10-phenanthroline reagents to give an orange coloured chromogen, measured at 510 nm. The MIC value for standard linezolid was found to be 2.7 µg/ml. It was observed that linezolid is unstable in acid and alkali. The potency of linezolid is critically reduced under these stress conditions, whereas under neutral, thermal and photolytic stress conditions the potency of linezolid was reduced to a smaller extent.

Keywords: Linezolid, Turbidimetric method, Spectrophotometric, Degradation.

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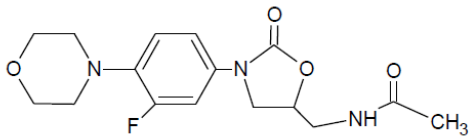
1. INTRODUCTION

The oxazolidinones are a new class of antimicrobials with good activity against gram positive bacteria. Antimicrobial resistance is a significant nosocomial problem and is of increasing importance in community-acquired infections. Linezolid, (*S*)-*N*-[[-(3-(3-fluoro- 4 (4- morpholinyl) phenyl]-2-oxo-5-oxazolidinyl]-methyl] acetamide (**Fig. 1**), is a synthetic compound that acts by inhibiting the formation of initiation complex in bacterial protein synthesis, a mechanism of action which is distinct from that of any other antibiotics that are commercially available[1]. It is available for oral administration as film-coated compressed tablets containing 600 mg linezolid. Literature survey revealed several methods reported for the estimation of Linezolid alone or in combination with other agents based on different techniques, RP-HPLC[1-6], LC-MS-MS[7], UV[8], HPTLC[9], Chiral HPLC[10], Spectrophotometric method [11]. Assays reported in the literature for the determination of linezolidin biological fluids include HPLC using UV-detection [12-15], and fluorescence detection [16]. However, only one stability indicative microbiological assay, using cup- plate method has been reported for potency determination of linezolid in the presence of photodegradation products [17].



Linezolid

Fig. 1: Chemical Structure of Linezolid.

2. MATERIALS AND METHODS

2.1. Instrument

A Labtronics Digital-LT-12 colorimeter was used for measuring the optical density in the microbial method. A Labindia Analytical UV 3000 double beam UV/Visible spectrophotometer with spectral bandwidth of 1 nm and a pair of matched quartz cells were used for measuring the absorbance in the spectrophotometric method.

2.2. Materials

All the chemicals and reagents used in the analysis were of analytical grade. Gift sample of standard Linezolid was kindly provided by Symed Laboratories Hyderabad India.

2.3. Preparation of culture suspension

The strain of *Staphylococcus aureus* ATCC 6538P were cultivated and maintained on Nutrient agar in the freezer and pealed to Muller Hinton broth (24h before the assay) that was incubated at 35±2.5°C for 24h. The bacteria, previously incubated in Muller Hinton broth, was also diluted with Muller Hinton broth to achieve a suspension turbidity of 25±2% using a colorimeter at wavelength 600 nm.

2.4. EXPERIMENTAL

2.4.1.Determination of Minimum Inhibitory Concentration (MIC) of standard linezolid drug

Standard linezolid stock solution of concentration 512 µg/ml was prepared by dissolving 0.512 mg of linezolid in 10 ml sterile distilled water. 14 sterilized test tubes were arranged in a rack. To each, 4 ml of sterilized Muller Hinton broth was added aseptically. A volume of 2 ml of standard linezolid stock solution was added aseptically to the first test tube. The test tube contents were cyclomixed and a volume of 2 ml was taken from it aseptically and transferred to the second test tube to give concentration of 256 µg/ml in the first test tube. In this manner serial dilution was continued till the twelfth test tube to give concentration of 0.25 µg/ml. All the test tubes were well cyclomixed. Then 0.1 ml of 24h old *S. aureus* culture (previously O.D adjusted to 0.1 at 600 nm) was added aseptically in all test tubes except the negative control test tube. Control tubes were prepared using the same procedure. To positive control 4ml of sterilized Muller Hinton broth, 4 ml of sterile distilled water and 0.1 ml of 24 h old *S. aureus* culture suspension was added. To negative control 4 ml of sterilized Muller Hinton broth and 4 ml of sterile distilled water was added without adding the culture. All the test tubes were again cyclomixed and then incubated at 35°C for 24 h. After 24 h the test tubes were checked for bacterial growth by measuring the optical density (O.D) of the solution at 600 nm using a colorimeter against the control. The results of MIC range is given in **Table 1**.

Concentration of drug (µg/ml)	Log concentration.	O.D at 600 nm*
Negative control		0.00
256	2.40	0.00
128	2.10	0.01
64	1.80	0.01
32	1.50	0.01
16	1.20	0.01
8	0.90	0.01
4	0.60	0.01
2	0.30	0.04
1	0	0.11
0.5	-0.30	0.12
0.25	-0.60	0.17

(*Average of three determination)

Table 1: Results of MIC range study

The plot of O.D v/s Log concentration is shown in **Fig. 2**.

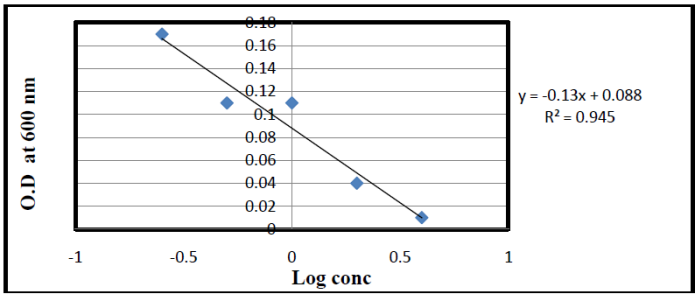


Fig. 2: Plot of O.D v/s Log concentration

2.4.2. Determination of exact MIC of linezolid

Standard linezolid stock solution was prepared by dissolving 10 mg of linezolid powder in 10 ml of sterile distilled water to give concentration of 1 mg/ml solution (1000 µg/ml). Working stock solution of linezolid was prepared by diluting 0.8 ml of the standard stock solution to 10 ml with sterile distilled water. 12 sterilized test tubes were arranged in a rack. To each, 4 ml of sterilized Muller Hinton broth was added aseptically. Different concentrations of linezolid solution were obtained by diluting a required volume of working stock solution with required volume of sterile distilled water.

After the addition of drug solution, the test tubes were cyclomixed and 0.1 ml of 24 h old *S. aureus* culture (previously O.D adjusted to 0.1 at 600 nm) was added aseptically in all test tubes except the negative control test tube (12th test tube). The test tubes were cyclomixed and then incubated at 35°C for 24 h. After 24 h the test tubes were checked for bacterial growth by measuring the optical density (O.D) of the solution at 600 nm using a colorimeter against the negative control. The results of MIC study for linezolid is given in **Table 2**.

Concentration of linezolid (µg/ml)	Volume of stock solution (ml)	Volume of diluent (ml)	Total volume (ml)	O.D at 600 nm*
2	1	3	4 ml	0.04
2.2	1.1	2.9		0.03
2.5	1.25	2.75		0.02
2.7	**1.35**	**2.65**		**0.01**
2.9	1.45	2.25		0.01
3	1.5	2.5		0.01
3.2	1.6	2.4		0.01
3.5	1.75	2.25		0.01
3.7	1.85	2.15		0.01
3.9	1.95	2.05		0.01
4	2	2		0.01

(*Average of three determination)

Table 2: Exact MIC results for linezolid

2.4.3. MIC test on stress degraded linezolid samples

Acid hydrolysis

To 10 ml of standard stock solution (1 mg/ml) of linezolid, 15 ml of 1N HCl was added in to a 50 ml volumetric flask. The flask was plugged with cotton and kept in water bath for 6 h at 70ºC. The sample solution was cooled to room temperature and was neutralized with a calculated volume of 1N NaOH to pH of 7. . The working stock solution of concentration 8 µg/ml was used for determining MIC of the acid degraded drug with *S. aureus* as test organism.

2.4.4. Alkali hydrolysis

To 10 ml of standard stock solution (1 mg/ml) of linezolid, 15 ml of 0.1N H NaOH was added in to a 50 ml volumetric flask. The flask was plugged with cotton and kept in water bath for 6 h at 70ºC. The sample solution was cooled to room temperature and was neutralized with a calculated volume of 0.1N HCl to pH of 7. The working stock solution of concentration 8 µg/ml was used for determining MIC of the alkali degraded drug with *S. aureus* as test organism.

2.4.5. Neutral hydrolysis

To 10 ml of standard stock solution (1 mg/ml) of linezolid, 15 ml of sterile distilled water was added in to a 50 ml volumetric flask. The flask was plugged with cotton and kept in water bath for 6 h at 70ºC. The sample solution was cooled to room temperature. The working stock solution of concentration 8 µg/ml was used for determining MIC of the neutral degraded drug with *S. aureus* as test organism.

2.4.6. Thermal degradation

20 mg of standard linezolid drug powder was placed in a glass petri dish. The petri dish containing the drug was kept in an oven for 6 h at temperatures 100°C. The working stock solution of concentration 8 µg/ml was used for determining MIC of the thermal degraded drug with *S. aureus* as test organism.

2.4.7. Photodegradation

20 mg of standard linezolid drug powder was placed in a glass petri dish. The petri dish containing the drug was kept in an UV chamber at 254 nm for 12 h. The working stock solution of concentration 8 µg/ml was used for determining MIC of the photolytic degraded drug with *S. aureus* as test organism.

2.4.8. Calculation of percent degraded drug at MIC concentration 2.7 µg/ml

As per the plot of log OD v/s concentration of standard linezolid in µg/ml, the trend line equation was found to be y = - 0.13x + 0.088, R^2= 0.945. The O.D seen for degraded linezolid sample at MIC concentration 2.7 µg/ml was substituted as the y value to find the concentration of active linezolid presents (value of x).

3. RESULTS AND DISCUSSION

The MIC value of standard linezolid is 2.7 µg/ml. i.e. the minimum inhibitory concentration of linezolid required to inhibit the growth of *S. aureus* is 2.7 µg/ml as no bacterial growth was seen at this concentration of linezolid. MIC study for degraded linezolid solution was done in concentration ranging from 0.25 µg/ml to 4.5

µg/ml. This range was selected because there was a possibility that the degradants that would be formed could show inhibitory action at concentration lower than the standard linezolid MIC value. **Table 3.** shows the O.D value at MIC conc 2.7 µg/ml of linezolid under different stress conditions.

Degradation condition	O.D value at MIC 2.7 µg/ml *
Acid **6hr**	0.12
Alkali **6hr**	0.15
Neutral **6hr**	0.07
Thermal **100 °C 6hr**	0.04
Photolytic **254 nm 12 hr**	0.04

(*Average of three determination)

Table 3. The O.D value at MIC 2.7 µg/ml of linezolid under different stress conditions

Comparison of Results for study of linezolid stress degradation samples by Spectrophotometric Method v/s Biological Method

The results obtained with the MIC method were partly comparable with the results obtained from the spectrophotometric method. In certain conditions significant difference was seen between the results of the two methods. It has been observed generally that linezolid is unstable in acid, alkali and oxidation stress condition. The potency of linezolid is critically reduced under these stress conditions. Whereas neutral, thermal and photolytic stress conditions reduced the potency of linezolid to a smaller extent. **Table 4.** shows the results of linezolid content after degradation studies by spectrophotometric method and microbiological -turbidimetric method.

Stress condition	time duration of stress study	Conc. Of linezolid by ferric citrate method	O.D at 2.7µg/ml MIC conc. of standard linezolid	Conc. Of linezolid from graph µg/ml (MIC method)	Per cent degradation seen by microbiological method (%)	Per cent degradation seen by spectrophotometric method (%)
Acid	6 hr	2.6	0.12	0.56	79.26	73.53
Alkali	6 hr	1.5	0.15	0.33	87.78	85
Neutral	6 hr	6.7	0.07	1.37	49.26	32.28
Thermal 100°C	6 hr	8.3	0.04	2.33	13.71	17
Photolytic	12 hr	8.3	0.04	2.33	13.71	16.6

Table 4: The results of Linezolid content after Degradation studies by Spectrophotometric method and Microbiological - Turbidimetric method

'Extreme parallel trend lines' (overlapping) could not be observed when amount of linezolid present in stress degraded solutions were determined by the two new methods, a spectrophotometric method and a turbidimetric antibiotic assay. The results of the two have been compared and are graphically presented as above where in, the efficacy of linezolid to inhibit the growth of microorganism was found to be about 5 to 10% lesser when hydrolytic degradation was carried out in acidic and alkaline medium, in comparison to spectrophotometric assay. Under neutral conditions of hydrolysis, the parallel trend could not be seen and variation of results was wider. However under thermal and photolytic conditions, the trend 'reversed' as the therapeutic efficacy was found to be better than the spectrophotometric assay by about 5%. This indicated a possibility that some degradants formed under thermal and photolytic stress conditions could be potentiating the antibacterial like activity, inspite of linezolid undergoing degradation - as determined and found from spectrophotometric assay. **Fig. 3**. represents trend lines comparing the amount of linezolid in solutions after degradation study.

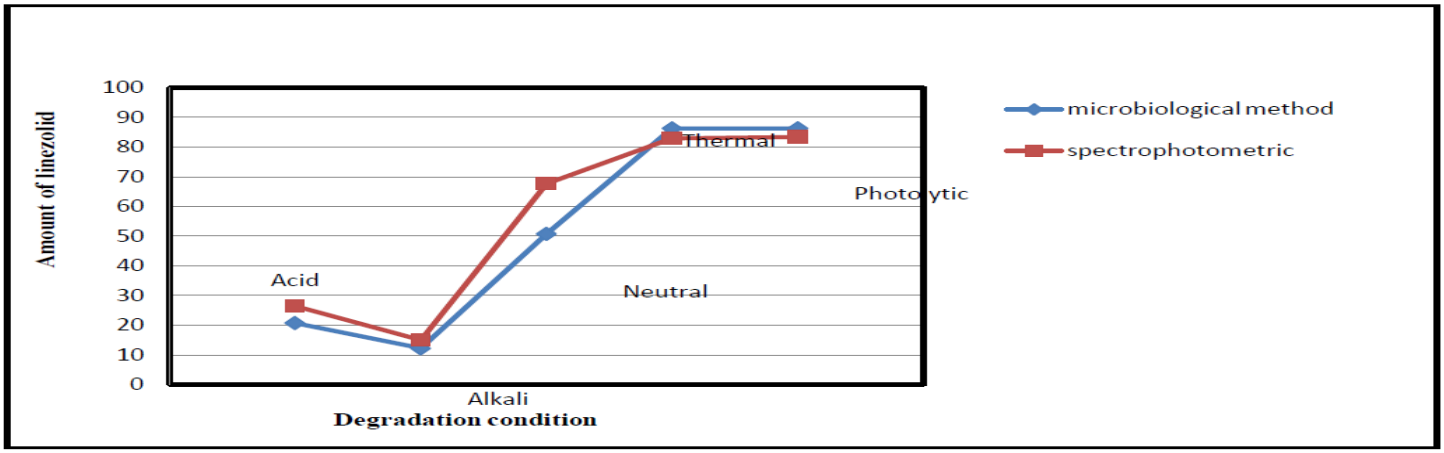


Fig. 3: Trend lines comparing the amount of Linezolid in solutions after degradation study.

4. CONCLUSION

The quantification of antibiotic components by chemical methods such as spectrophotometry cannot provide a true indication of microbial activity. The microbiological test evaluates the potency of the drug and spectrophotometers not always detects changes in the structure of the molecule, so importance of microbiological test should be held in conjunction with a physical- chemical method.

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