Validation of Microbiological Assay Design of Neomycin Sulfate in 30 x 30 cm Rectangular Antibiotica Plate

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Abstract

The microbiological assay is one of the most commonly used techniques for potency determination using biological means through indicator microorganisms in research, clinical, industrial and quality control activities. The current work aimed to validate the determination of Neomycin Sulfate potency through the agar diffusion method using the 2 x 2 Parallel Line Model. The study investigates both the quality of results as a zone of inhibition and the inspected validation parameters which consist of specificity, linearity, accuracy, precision (in terms of repeatability and intermediate precision) and robustness. The initial step determined the validity of raw data for further analysis in terms of normality using QQ plot, homoscedasticity by Cochran’s test and the absence of true outlier values through both G statistics (USP), Robust regression and Outlier removal tests. The assay design and procedure showed selectivity towards the intended antibiotic and the calibration curve showed acceptable linearity (r = 0.998) without any fixed or relative concentration-related bias. Accuracy profile covering 50, 100 and 150% of the target concentration demonstrated potencies with a confidence interval that is contained within the acceptance limits. Results of the precision and robustness showed RSD% <2.00. The assay design is suitable for the assay of Neomycin Sulfate.

Keywords
Neomycin Sulfate, Linearity, Accuracy, Precision, Robustness
Introduction

Antimicrobial drugs are one of the important and widely consumed medications that are used in combating microbial infection which impact human health may be life-threatening in extreme cases [1,2]. The safety and efficacy of medicinal products are crucial requirements to achieve the target aim from their use without losing therapeutic value and with minimal toxicity exposure [3]. Aminoglycoside antibiotics are one of the important classes of antimicrobials that found their use in topical and parenteral administration [4,5]. Neomycin Sulfate is one of these antimicrobials which are listed in the well-known national and international pharmacopeia [6].

The potency of the Active Pharmaceutical Ingredient (API) - which is antibacterial - is determined by microbiological antibiotic assay using the zone of inhibition technique [7,8]. There is a limited linear relationship between the biological effect (i.e. clear halo diameter) and the concentration of the antibiotic or its transformed dose levels [9]. This linearity is found within relatively small range of the antimicrobial concentration and appropriate determination of this limitation is important in the design of Parallel Line Model (PLM) when the potency of the sample should be determined [10].

In order to quantify the biological potency of the antimicrobial compounds and obtain reproducible results with reasonable quality and confidence an appropriate antibiotic assay design should be established and examined for its suitability and must be validated to ensure that the proposed design has fulfilled its target and met the validation acceptance criteria [11]. Thus, it can be used for routine testing of that antibiotic either as a raw material or incorporated in formulated consumable products for medical use.

In the present work, a validation study of a 2 x 2 balanced PLM design was conducted for Neomycin Sulfate antibiotic. The assay layout involved large 8 x 8 rectangular plates with dimensions of 30 x 30 cm. The validation criteria embraced specificity, linearity, precision (repeatability and intermediate precision) and robustness, in addition to the statistical analysis of the output datasets validity for potency determinations.

Material and Methods

The selected antibiotic design was a 2 x 2 balanced PLM in large rectangular 30 x 30cm that accommodate 8 columns x 8 rows of paper discs, wells or cylinders. The method applied for the analysis of the potency determination of Neomycin Sulfate followed the United States Pharmacopeia (USP) for procedures, media, chemicals and reagents [12]. The modification herein included the use of Bacillus pumilus (National Collection of Industrial Bacteria (NCIB) 8982) as the test microorganism prepared from a freshly grown microbial culture, well-spread over the surface of antibiotic medium #1 and incubated at 36 ±1°C for 21 ± 3 hours. Microbial suspension for the assay was prepared at the end of the incubation period as a working stock suspension by harvesting the microbial growth from the agar surface in a sterile saline or buffer solution. The test organism density was adjusted at 5800 Angstroms (Å) and absorbance range of 0.6505 ± 0.0485 Absorbance Units (AUs). Treatment groups – High (H) dose and Low (L) dose - were applied in the plates using some sort of randomization such as Latin square for each preparation (Standard (S) and Test (T) or Unknown (U)) [13,14]. The validation parameters were selectivity, linearity, precision (intermediate precision and repeatability) and robustness (medium pH and incubation time) [15]. The assay suitability was assessed using statistical analysis of raw data (unscrambled and grouped) through previously programmed and validated spreadsheets [16].

1. Statistical Analysis of Raw and Arranged Datasets

Row and column totals of the plates were investigated visually for homogeneity without the presence of the aberrant pattern [17]. Readings of the arranged data of inhibition zones (mm) in columns as treatment groups were investigated statistically in terms of normality, homoscedasticity (homogeneity of variance), outliers and Analysis of Variance (ANOVA) for regression and parallelism with computed probabilities for each conducted experiment (p = 0.05, while for regression p = 0.001) [18]. All complex calculations were programmed in Excel sheets and were verified for validity using model examples previously calculated [16,19]. The calculation included concurrent determination of the potency and 95% confidence interval [17,18]. These statistical tests were used complementarily with the validation parameters to ensure the suitability of datasets and the validity of the assays for potency determinations.

2. Validation Parameters for Microbiological Antibiotic Assay

The selected assay design was investigated in terms of specificity, linearity, precision (repeatability and intermediate precision) and robustness to ensure the validity of the assay layout for potency determination of Neomycin Sulfate [20-23].

2.1. Specificity

Selectivity of the assay design and procedure toward the dedicated API was investigated through conducting full experimentation with a positive group against a negative control group that included all constituents of the product without Neomycin Sulfate in the formulation (Placebo) [19]. No detectable zone of inhibition should be observed in the assay plate for the negative control, in contrast to the positive Neomycin Sulfate control group [19]. No interference should occur from any reagents, chemicals or other biologically active/excipients of the product.

2.2. Linearity

Calibration curve was constructed using different logarithmically transformed dose levels of the antibiotic in x-axis against the response in y-axis expressed as zone of inhibitions diameter (mm) [24]. The examined concentration range was 10 to 30 µg/ml. Linearity was evaluated through linear regression analysis by correlation coefficient and the regression significance [25]. It was verified that the methods present linearity when the correlation coefficient (r) is bigger or equal to 0.90 and the regression significance is inferior to 0.01 [25]. It could be concluded that the linearity curve is acceptable, that is, it does not present fixed or relative tendency when the residual plot for linear and angular coefficients include, respectively, value of zero [26-28]. The precision profile of the antibiotic concentration levels – through the Relative Standard Deviation precent (RSD%) – should show similar outcome.
2.3. Accuracy

The accuracy was determined by adding known amount of Neomycin Sulfate with known potency to the samples of the formulated product under investigation [20]. Accuracy was evaluated in relation to theoretical doses and was determined through accuracy profile analysis, by plotting theoretical concentrations versus experimentally determined relative error percent (RE%) [29]. The tolerance interval, concerning the accuracy of the 2 x 2 experimental design, was estimated through experimental determinations in three levels of concentration (30%, 100%, and 150% of the reference dose) in the medicinal formulated products. It has been concluded that the method is accurate, that is, the assay and the estimated confidence values from the calculated potencies of Neomycin Sulfate should be confined within the acceptance criteria at all concentration levels for accurate quantification of the dedicated API [25, 29].

2.4. Precision and Robustness

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay) [20]. Repeatability was evaluated by assaying three samples at the same concentration and under identical working conditions. The intermediate precision was verified by comparing the assays on two different occasions. The precision is calculated as RSD% [30, 31]. The robustness of the method was determined by analyzing the same sample under a variety of conditions [22]. The significant factors considered – herein in this study - were incubation time and pH of the antibiotic medium through small modifications. The test samples were assay against the Neomycin Sulfate standard material of known activity. The obtained responses were evaluated according to the RSD calculated among the experimental groups [23]. The RSD% values obtained were investigated to be lower than 5%

Results and Discussion

Neomycin Sulfate is an aminoglycoside antibiotic that consists of a mixture of five related microbiologically active compounds [32]. The proportion of these materials are fairly variable between different commercial products of this API [32]. While several chemical analysis techniques involving rapid and automated methods such as HPLC could be developed, yet the microbiological assay using inhibition zone retains its value as a cheap, simple and safer approach as it does not include complex instrumentation, toxic or hazardous chemicals [11]. Moreover, the true combined antimicrobial effect would be easily measured using the biological method. This is despite the fact that individual active components might be determined with high precision and accuracy using chemical means.

Official international monographs showed detailed procedures for conducting microbiological potency determinations for compendial antimicrobial materials listed under those tested using the antibiotic assay technique [12, 32]. However, each laboratory should select a convenient layout design that is deemed suitable for the aim of this activity and the nature of this work. However, the appropriately selected design should be validated to ensure its suitability for activity determination with reasonable confidence [33]. The investigated parameters in the current validation study embrace the selectivity, linearity, accuracy, precision (repeatability and intermediate precision) and robustness, in addition to the examination of raw data suitability and the validity of the conducted test.

Preliminary Dataset Assessment

Before conducting complex statistical analysis and result interpretation for the assay, the initial raw data should be reviewed for suitability of further processing [16, 18]. Examining unscrambled data for row and column totals of each conducted experiment should return homogenous outcome without any aberrant pattern which was observed in this study through calculation in a previously programmed spreadsheets [18]. Moreover, four assumptions must be basically fulfilled for a valid assay, namely: linear dose-response relationship and ensuring randomization allocation of groups across the assay plate [17]. Other aspects that should be investigated were reasonable normality of the recorded data distribution, true outlier detection/omitting and homogeneity of variances [17].

1. Examination of Data Normality

Results of the inhibition zone diameter for each treatment group were checked for normality by both Anderson-Darling (AD) and Shapiro-Wilk (SW) tests [33]. The SW test described in the pharmacopeia is for groups of seven or more [17]. Figure 1 demonstrates the QQ normality plot for all treatment groups conducted in the validation of the experimental design [34]. Data points are close to the ideal line assumed for Gaussian distribution with further confirmation executed using normality tests. Deviations from normality are not likely and, in any occasion, do not seriously jeopardize the assay. Nevertheless, the European Pharmacopeia (EP) suggests that in case of doubt the SW test might be used to spot any deviations from normality [35]. It is not glib to be certain of revealing drifts in the normality of distribution by inspection in the case of a few replicates [17]. The author highlighted that it seems reasonable to assume that in a well-conducted microbiological assay, responses will be normally distributed unless there are outliers, which should be eliminated from the calculation after reasonable justification.

2. Detection of Outlier Values

One of the important screening procedures of antibiotic assay zone of inhibition reading is the examination of the presence of outlier values in the datasets as this may affect the normality of the inspected group. When using G statistics by USP method for spotting aberrant values, almost all treatments groups did not exceed the critical value limit from either upper (U) or lower (L) extreme values [16]. Exceptions could be found in two treatment groups in two experiments. However, rejection of an excursion that resides merely on this test alone might result in false omitting of a true valid value, especially in the absence of obvious justification for removing this excursion after careful investigation of the assay procedure and the zone pattern across the plates [17]. A thorough examination of data trends and experience should play a confirmatory role in decision-making to avoid unwanted bias in data analysis. One reason that could be playing a role in this event might be the clustering tendency that would be observed using a box plot, where one or more reading(s) stood off other aggregated data [17, 33]. On the same line, no outliers could be detected and removed using the Robust regression and Outlier removal (ROUT) combination test.
3. Test of Homoscedasticity (Homogeneity of Variance)

This inspection characteristic - homogeneity of variance (homoscedasticity) - might be checked by either Bartlett’s test or Cochran’s test [17]. However, it is noteworthy that Bartlett’s procedure was criticized by Box (1953) as being very sensitive to non-normality. So, Bartlett’s test was not used in the present analysis [36]. Figure 2 illustrates the implementation of the homogeneity of variances test. None of the experimental validation groups showed Cochran’s value (blue dots) that exceeds the critical limiting value (red dashed line) at α = 0.05. All tests in the 2 x 2 PLM assay design showed homogenous variances. Thus, the homoscedasticity condition was fulfilled. No evidence of abnormality in the raw data could be detected. Accordingly, validation of the assay design was conducted by inspecting the necessary parameters of specificity, linearity, accuracy, precision and robustness.

Specificity of Antibiotic Assay Design

To ensure that the assay procedure is selective toward the dedicated API only, a full and exact analysis process with conducted using postulated 2 x 2 PLM design in the large rectangular assay plate but without inclusion of Neomycin Sulfate antibiotic in the test group components [19]. Absence of any detectable zone of inhibition from the test samples in the presence of well-defined inhibition zones from the positive control group is an indication of the selectivity of the designed layout and procedure of the investigated balanced assay.

Linearity of Antibiotic Assay Design

A calibration curve was constructed using five concentration levels for the zone of inhibition (in mm) vs. logarithmically transformed concentrations [34]. The plot showed acceptable linearity with r = 0.998 as could be found in Figure 3. The precision diagram is demonstrated in Figure 4 without any significant sign of either fixed or relative bias. The angular coefficient included a zero value without any detectable trend associated with the concentration [37]. The same outcome could be observed in Figure 5 with the residual plot after evaluation of both angular and linear coefficients. Thus, the inspected concentration range would reasonably cover the required linear requirement of the PLM assay of Neomycin Sulfate antibiotic. For a total Degree of Freedom (df) 4, Sum of Squares (SS0 was 28.65238, with residual df = 3, SS = 0.13466 and Mean Square (MS) = 0.04489. The regression analysis with a df of one showed SS and MS = 28.51772, F = 635.3315 and F-significance = 0.00014 which is lower than 0.01 value.

Accuracy of Antibiotic Assay Design

The accuracy was proved by recovery tests that are designed experiments to ensure the agreement between the actual values found for the analyte and the true added amount of that analysis [20-22]. The recovery test was performed at three different concentrations. The reported results obtained from the bioassay were close to the true concentration values of the samples. The method had appropriate accuracy, as can be seen by the values calculated for the β-tolerance interval (Figure 6) for each concentration level, which showed a maximum coefficient of variation not exceeding 15% for concentration range including 50, 100 and 150% [29, 38]. The mean potency was shown at each concentration level as square dots in the graph. Accuracy is represented by the combination of the random (precision) and systematic (trueness) errors, which were considered in the β-tolerance interval calculation [29, 39]. Relative bias and recovery were calculated to assess the trueness of the method. This represents the interval in which β% of the future individual results is expected [40]. As shown in this figure, the tolerance limits remained within the acceptance limits - including tighter upper limit for parenteral preparations - on the whole, experimental concentration range for the analyte. Thus, the accuracy of the microbiological method was demonstrated over investigated concentration levels.

Figure 1: QQ normality plot showing the distribution trend of datasets of each treatment group in validation study campaign
Figure 2: Homoscedasticity of potency determination test groups for accuracy, repeatability (R), intermediate precision (I), control, pH and the incubation period (IP) showing homogeneity of variance with limiting value demonstrated by red dashed line at $\alpha=0.05$.

Figure 4: Precision-concentration association diagram. The linear regression analysis did not demonstrate a significant difference of precision between the levels evaluated, once the angular coefficient included zero value.
Figure 3: Linearity curve of Neomycin Sulfate showing average reading points of inhibition zones (mm) with standard deviations against logarithmic transformed five concentrations (µg/ml). The linear equation is shown along with the coefficient of determination value.

Figure 6: Accuracy profile obtained for the method of microbiological dosage of Neomycin Sulfate for 2 x 2 design in a large rectangular plate. Solid lines represent acceptance limits (-22.5%, 22.5%), the dotted line is the tightest upper specification limit which is found in compendial reference whereas dashed lines represent 95% tolerance interval reached. When tolerance intervals are located within the acceptance limits, the assay can be quantified accurately.
Figure 5: Residual plot showing the absence of fixed or relative bias through the examined concentration range of Neomycin Sulfate evaluated through angular and linear coefficients, which included value zero

Table 1: Precision and robustness of 2 x 2 antibiotic assay design of Neomycin Sulfate in 30 x 30 cm rectangular plate

| Sample  | Concentration (mg/g) | Mean total diameter of inhibition zone (mm) ± midrange | Intra-assay inhibition zone RSD (%) | Intra-assay RSD (%) | Confidence interval percent (95%) | Mean sample potency (%) | Inter-assay RSD (%) |
|---------|----------------------|--------------------------------------------------------|------------------------------------|---------------------|----------------------------------|------------------------|---------------------|
| Control | 2.79                 | 28.14 ± 2.93                                           | 1.35                              | 28.14 ± 2.93        | 1.35                             | 28.14 ± 2.93           | 1.35                |
|         | 2.84                 |                                                        |                                    |                     |                                  |                        |                     |
| I       | 2.78                 | 28.37 ± 3.21                                           | 1.51                              | 28.37 ± 3.21        | 1.51                             | 28.37 ± 3.21           | 1.51                |
|         | 2.75                 |                                                        |                                    |                     |                                  |                        |                     |
| II      | 2.79                 | 28.30 ± 2.40                                           | 1.66                              | 28.30 ± 2.40        | 1.66                             | 28.30 ± 2.40           | 1.66                |
|         | 2.78                 |                                                        |                                    |                     |                                  |                        |                     |
| III     | 2.79                 | 28.30 ± 2.40                                           | 1.68                              | 28.30 ± 2.40        | 1.68                             | 28.30 ± 2.40           | 1.68                |
|         | 2.81                 |                                                        |                                    |                     |                                  |                        |                     |
| A       | 2.77                 | 28.37 ± 3.21                                           | 1.51                              | 28.37 ± 3.21        | 1.51                             | 28.37 ± 3.21           | 1.51                |
|         | 2.79                 |                                                        |                                    |                     |                                  |                        |                     |
| B       | 2.74                 | 29.47 ± 2.38                                           | 1.56                              | 29.47 ± 2.38        | 1.56                             | 29.47 ± 2.38           | 1.56                |
|         | 2.77                 |                                                        |                                    |                     |                                  |                        |                     |
| pH      | 2.84                 | 29.47 ± 2.38                                           | 1.69                              | 29.47 ± 2.38        | 1.69                             | 29.47 ± 2.38           | 1.69                |
|         | 2.77                 |                                                        |                                    |                     |                                  |                        |                     |
| Incubation Time | 2.81       | 28.18 ± 2.91                                           | 1.57                              | 28.18 ± 2.91        | 1.57                             | 28.18 ± 2.91           | 1.57                |
|         | 2.78                 |                                                        |                                    |                     |                                  |                        |                     |

* Calibrated digital caliper was used in reading the diameter of inhibition zones to two decimal digits sensitivity

** If any outlier in the original dataset of inhibition zone was detected by exceeding G critical value, the record was examined first to exclude false rejection of truly acceptable value based on experience, trending of data and additional statistical testing using ROUT combination (GraphPad Prism V6)

$ For each dose level (high and low) 16 measurements were made for each preparation

§ In case of missing or excluded values, the rule of replacement reading was applied to maintain the assay balance without impacting the output

£ Relative Standard Deviation within an assay

€ Relative Standard Deviation between different assay determinations for potency
Precision and Robustness of Antibiotic Assay Design

The overall RSD% for all assay groups was acceptable with a value of 0.71. Table 1 shows detailed results of the precision (repeatability and intermediate precision) and robustness. Moreover, RSD% was determined to assess the variation in the inhibition zone within the experiments and to evaluate potency determinations (expressed as %). On the other hand, the Confidence Intervals (CIs) - at 95% - showed the calculated range of the upper and the lower values that most likely would contain the true population values of the potency. The output was evaluated through RSD% computed within and between experimental campaign design [20]. The variability between inhibition zones within individual assay plates was calculated. In the microbiological assay, the number of replications per dose must be sufficient to ensure the desired precision with acceptable confidence [20]. Furthermore, the assay may be repeated and the results combined statistically to obtain the required precision [12, 20]. The Control group was included for the comparative study with an average value of 2.82 mg/g, RSD% of 1.26 and relative error of -0.09%. CI estimates were included and expressed as a percentage. Repeatability test showed an RSD value of 0.71% with an average of 98.96%, 1.04% relative deviation from control and relative error of 1.12%. Intermediate precision demonstrated an RSD value of 0.74% with an average of 98.40%, 1.60% deviation from the control group and relative error of 1.69%. On the other hand, robustness demonstrated an RSD value of 1.13% with an average of 99.56%, deviation from the standard of 0.44% and relative error of 0.53%. The 95% CIs (expressed as a percent) showed that the forecasting for the ranges of potency estimates was reasonably acceptable for the assay. Nevertheless, increasing the number of replicates per treatment group would increase the confidence according to the desired target needed.

Analysis of Variance for Regression and Parallelism

Statistical evaluation of inhibition zone data must be ensured before deriving any conclusion or confirming output results from the assay design. Two official criteria must be met to ensure the suitability of assay data to compute the potency of Neomycin Sulfate [17]. These parameters are regression and parallelism which were verified using Analysis of Variance (ANOVA) as could be seen in Table 2. In case of missing and/or rejected values from the assay readings, the degree of freedom would change and hence F-tabulated values could change [17]. In addition, the probability of the occurrence of the events in terms of meeting the limiting criteria was also calculated. The roles of replacing missing values were applied so that only the assay balance was resumed without affecting the original output record [12]. When appropriate randomization procedures were applied through the assay plate, the effect of row and column variations could be minimized [17]. Detailed statistical analysis of the mandatory compendial parameters showed an acceptable regression and parallelism by comparing F-calculated (derived from the sum of squares, mean square and degree of freedom (d.f.)) with F-tabulated, in addition to the probabilities of the computed events. All the results of the zone of inhibition assay design had met the acceptance criteria for both regression and parallelism. Hence, all experiments were valid and suitable to derive the potency values for the samples.

Conclusion

Microbiological antibiotic assay of Neomycin Sulfate using 2 x 2 balanced PLM agar diffusion technique in large 30 x 30 cm large rectangular (8 rows x 8 columns) antibiotic plates was validated in terms of specificity, linearity, accuracy, precision and robustness, in addition to the examination of datasets suitability and assay design validity for potency determinations of the aminoglycoside antibiotic. The investigated design showed the acceptable design and validation parameters. Thus, it is suitable for the assay with reasonable confidence. Shall the confidence window be needed to get more restricted, an assay modification that includes an increase in the number of replicates must be investigated.

Conflict of Interest

There is no competing conflict of interest between the authors.

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Table 2: Analysis of Variance (ANOVA) involving official mandatory criteria for assay suitability

| Source of Variance | Validation Group | Sum of Squares | Mean Square | Variance Ratio | Calculated Probability |
|--------------------|------------------|----------------|-------------|----------------|------------------------|
|                    |                  | Accuracy       |             |                |                        |
|                    | 50%              | 116.04         | 116.04      | 145.67         | <0.0001                |
|                    | 100%             | 132.52         | 132.52      | 598.05         | <0.0001                |
|                    | 150%             | 109.28         | 109.28      | 501.60         |                        |
|                    | Repeatability    |                |             |                |                        |
|                    | I                | 212.69         | 212.69      | 1023.41        | <0.0001                |
|                    | II               | 150.07         | 150.07      | 764.90         | <0.0001                |
|                    | III              | 147.08         | 147.08      | 811.91         |                        |
|                    | Intermediate Precision |          |             |                |                        |
|                    | A                | 212.68         | 212.68      | 1657.93        | <0.0001                |
|                    | B                | 356.97         | 356.97      | 1561.10        |                        |
|                    | Robustness       |                |             |                |                        |
|                    | Control          | 214.77         | 214.77      | 2184.71        | <0.0001                |
|                    | Incubation Time  | 205.42         | 205.42      | 1213.39        | <0.0001                |
|                    | Medium pH        | 124.04         | 124.04      | 442.03         |                        |
|                    | Accuracy         |                |             |                |                        |
|                    | 50%              | 2.12           | 0.71        | 0.89           | 0.4993                 |
|                    | 100%             | 1.36           | 0.45        | 2.05           | 0.1400                 |
|                    | 150%             | 0.83           | 0.28        | 1.28           | 0.3345                 |
|                    | Repeatability    |                |             |                |                        |
|                    | I                | 0.08           | 0.03        | 0.30           | 0.7170                 |
|                    | II               | 0.30           | 0.10        | 0.50           | 0.6760                 |
|                    | III              | 0.19           | 0.06        | 0.36           | 0.7151                 |
|                    | Intermediate Precision |          |             |                |                        |
|                    | A                | 0.50           | 0.17        | 1.30           | 0.3249                 |
|                    | B                | 1.32           | 0.44        | 2.55           | 0.0787                 |
|                    | Robustness       |                |             |                |                        |
|                    | Control          | 0.33           | 0.11        | 1.13           | 0.3892                 |
|                    | Incubation Time  | 0.31           | 0.10        | 0.60           | 0.6322                 |
|                    | Medium pH        | 0.68           | 0.23        | 0.80           | 0.5373                 |

*a* F-tabulated limiting value >12.52, 12.56, 12.61, 12.66 or 12.71 for d.f. of one and Residual Error (SSreg) d.f. of 42, 41, 40, 39 or 38, respectively

*b* F-tabulated limiting value <2.83, 2.84, 2.85 or 2.85 for d.f. of three and Residual Error (SSreg) d.f. of 42, 41, 40, 39 or 38, respectively

References

[1] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and therapeutics. 2015;40(4):277.
[2] Prestinaci F, Pezzotti P, pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and Global Health. 2015;109(7):309-318.
[3] World Health Organization. Safety of medicines: pharmacovigilance programme of India. WHO Drug Information. 2018;32(1):10-7.
[4] Krause K, Serio A, Kane T, Connolly L. Aminoglycosides: An Overview. Cold Spring Harbor Perspectives in Medicine. 2016;6(6):a027029.
[5] Block M, Blanchard DL. Aminoglycosides. StatPearls [Internet]. 2021.
[6] Adams E, Liu L, Dierick K. Guymard S, Nabet P, Rico S et al. Neomycin: microbiological assay or liquid chromatography?. Journal of Pharmaceutical and Biomedical Analysis. 1998;17(4-5):757-766.
[7] Dafale NA, Semwal UP, Agarwal PK, Sharma P, Singh GN. Development and validation of microbial bioassay for quantification of levofloxacin in pharmaceutical preparations. Journal of Pharmaceutical analysis. 2015;5(1):18-26.
[8] Dafale NA, Semwal UP, Agarwal PK, Sharma P, Singh GN. Quantification of ceftriaxone sodium in pharmaceutical preparations by a new validated microbiological bioassay. Analytical Methods. 2012;4(8):2490-8.
[9] Elliott RL. Antibiotics: A Multidisciplinary Approach By Giancarlo Lancini, Francesco Parenti, and Gian Gualberto Gallo. Plenum Press, New York. 1995.
[10] Fleetwood K, Bursa F, Yellowlees A. Parallelism in practice: approaches to parallelism in bioassays. PDA Journal of Pharmaceutical science and technology. 2016;1:4(2):248-63.
[11] Dafale NA, Semwal UP, Rajput RK, Singh GN. Selection of appropriate analytical tools to determine the potency and bioactivity of antibiotics and antibiotic resistance. Journal of Pharmaceutical analysis. 2016 Aug 1;6(4):207-13.
[12] USP-NF (81) Antibiotics—Microbial Assays. The United States Pharmacopeia, 43rd ed. Rockville, Md.: The United States Pharmacopeial Convention; 2021.
[13] Kirk RE. Latin square design. The Corsini Encyclopedia of Psychology. 2016 Aug 1;4(4):591-6.
[14] Rao T. Validation of Analytical Methods. Calibration and Validation of Analytical Methods - A Sampling of Current Approaches. 2018.
[15] Eissa DE, Rashed ER, Eissa ME. Suitability System of Microbiological Assays. The United States Pharmacopeia. 43rd ed. Rockville, Md.: The United States Pharmacopeial Convention; 2021.
[17] Hewitt W. Microbiological assay for pharmaceutical analysis. Boca Raton: Interpharm/CRC; 2004.
[18] Eissa M, R Rashed ER, Eissa DE. Statistical Comparison of Parallel-Line Symmetrical Microbiological Models: Analysis of Agar Diffusion Assay in 8 x 8 Large Rectangular Plates. Journal of Statistics and Applied Sciences. 2021 (In-Press).
[19] Eissa M, R Rashed ER, Eissa DE. Validation of Symmetrical Two-Dose Parallel Line Assay Model for Nystatin Potency Determination in Pharmaceutical Product. Journal of Advanced Pharmacy Research. 2021.
[20] Oppe TP, Menegola J, Schapoval EE. Microbiological assay for the determination of cefpirome in raw material and injectable preparation. Drug Analytical Research. 2018;2(1):29-35.
[21] Oppe T, Menegola J, Schapoval E. Development and Validation of UV Spectrophotometry and Liquid Chromatography Methods for Determination Of Cefpirome in Raw Material and Pharmaceutical Dosage. Drug Analytical Research. 2019;3(1):42-50.
[22] Cazedey EC, Salgado H. Development and validation of a microbiological agar assay for determination of orbifloxacin in pharmaceutical preparations. Pharmaceutics. 2011;3(3):572-81.
[23] Vierra D, Fiuza TF, Salgado H. Development and validation of a rapid turbidimetric assay to determine the potency of cefuroxime sodium in powder for dissolution for injection. Pathogens. 2014;3(3):656-66.
[24] Nahar S, Khutun M, Kabir M. Application of microbiological assay to determine the potency of intravenous antibiotics. Stamford Journal of Microbiology. 2020;10(1):25-29.
[25] Lourenço FR, Pinto TD. Comparison of three experimental designs employed in gentamicin microbiological assay through agar diffusion. Brazilian Journal of Pharmaceutical Sciences. 2009;45:559-66.
[26] Caulcutt R, Boddy R. Statistics for analytical chemists. London: Chapman and Hall/CRC; 1983.
[27] Beiguelman B. Curso prático de bioestatística. 5th ed.; Ribeirão Preto: Revista Brasileira de Genética; 2002; pp.1-35, 117-263.
[28] Box GE. Non-normality and tests on variances. Biometrika. 1953 Dec 1;40(3/4):318-35.
[29] Hubert P, Nguyen-Huu JJ, Boulanger B, Chapuzet E, Chiap P, Cohen N, Compagnon PA, Dewé W, Feinberg M, Lallier M, Laurentie M, Muzzard G. Harmonization of strategies for the validation of quantitative analytical procedures: A SFSTP proposal–part II. Journal of pharmaceutical and biomedical analysis. 2007;45(1):70-81.