# Microbiological Antibiotic Assay Validation of Gentamicin Sulfate Using Two-Dose Parallel Line Model (PLM)

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## Abstract

Till nowadays microbiological assay is still widely used with several antibiotics that are composed of a mixture of related active compounds. However, obtaining a reasonably valid determination of the potency is dependent on the validity and the suitability of the assay design. The present work aimed to validate an assay design of an aminoglycoside antibiotic (Gentamicin Sulfate) using a two-dose Parallel Line Model agar diffusion assay in a large 8×8 rectangular plate. All preparatory procedures were done following United States Pharmacopeia and the Inhibition Zones were measured using a digital caliper to the nearest 0.01 mm. Analysis of variance of compendial requirements of regression and parallelism were found to be satisfactorily meeting the acceptance criteria. Specificity was achieved for the product under investigation with no detectable IZ that could be found for all components except the antibiotic. The validation method showed acceptable linearity of $r^2 \geq 0.98$. Accuracy and precision parameters showed RSD (%)<2. All relative error value estimates were below 4%. The proposed validation design for 32×32 cm antibiotic plates yielded valid results and can be projected for the routine Quality Control analysis of the antibiotic material, especially which is incorporated into a finished medicinal dosage form.

*Keywords:* Gentamicin Sulfate; PLM; Regression; Parallelism; Linearity; Precision; Ruggedness; Agar Diffusion; Inhibition Zone.

## 1. Introduction

In the world of ever-growing populations with the compromised immune system and deficient heath, the administration of appropriate antimicrobial drugs becomes more important for treatment of infections or even lifesaving diseases in some cases [1]. Careful delivery of a reasonably accurate dosage to affected patients must be ensured to obtain the desired therapeutic value without toxicity or inefficiency from the administered medicinal products [2]. One of the important classes of antimicrobials is the aminoglycosides group of antibiotics [3]. These drug materials are produced naturally from microorganisms which produce a family of related active compounds that may comprise several microbiologically active constituents [3]. While modern techniques for analysis (such as HPLC and UPLC) are appealing and convenient for the assay of many compounds as could be found in the official monographs, yet they cannot give a true estimate for the antimicrobial activity of the active antibiotic ingredients in combination [4, 5].

For this reason, the microbiological antibiotic assay is still widely used for some antimicrobials to derive the actual potency through the biological inhibitory activity for the growth of certain microorganisms as listed in the compendial monographs [6]. Despite the great advances that have been achieved technologically in the procedures of this type of test, yet to date, it depends largely on manual operation, especially in developing countries [7]. In turn, this might influence the outcome of the test, notably in the routine activity monitoring for Active Pharmaceutical Ingredient (API) in final consumable products [8]. Due to the above challenges, the present work focused on the development of a simple validation system for the assessment of the antibiotic assay design to ensure its applicability in the frequent working activity for Gentamicin Sulfate using a two-dose Parallel Line Model (PLM) in large rectangular 8 x 8 assay plates. Therefore, the target of the present study was to develop and validate a low-cost, simple, specific, accurate and reproducible microbiological agar diffusion assay using an agar-well method and propose it as a useful technique for quantitation of Gentamicin Sulfate.

## 2. Materials and Methods

The devised layout of the assay was established in large rectangular sterilizable 32×32 cm plates. Each plate could accommodate 8 rows by 8 columns of sample discs, wells or cylinders. In each plate, an equal number of all treatments were used in a balanced layout. The selected design was a two-dose PLM model in (8 columns×8 rows) antibiotic assay plates using the zone of inhibition technique. The two-dose assay method was evaluated by the determination of linearity, accuracy, precision and [9].

### 2.1. Statistical Analysis for Gentamicin Sulfate Assay

The terms "mean or average" and "standard deviation or S.D." are used herein as defined in conventional current textbooks of biometry [10]. The other parameters are used in this study to indicate the experimental variation validity would be assessed using sum of squares, mean square and variance ratio, along with calculated probability to assess assay suitability to estimate potencies [11]. Assay outputs are stated to be "statistically valid" if the outcome of the analysis of variance (ANOVA) is as follows [12]:

- The linear regression term is significant: the calculated probability is not less than the limiting critical value. If this criterion is not met, it is not possible to calculate 95% confidence limits (CL).

- The term for non-parallelism is not significant: the calculated probability is less than F-tabulated for the hypothetical threshold. Otherwise, the assay would be invalid and should be repeated.

However, a significant deviation from parallelism in multiple assays may be due to the inclusion in the assay-design of preparation to be examined that gives a transformed (dose)-response line with a slope significantly different from those for the other preparations. Instead of declaring the whole assay test invalid, it may then be decided to eliminate or exclude all data relating to that questionable preparation and to restart the analysis from the beginning [12, 13]. When statistical validity is established, potencies and confidence limits may be estimated.

### 2.2. Specificity of Assay Design and Conditions

This test is aimed to show the ability of the microbiological assay to unambiguously assess the Active Pharmaceutical Ingredient (API) in presence of all other conventional commercial components in a market pharmaceutical-grade product for clinical use, in addition to other expected conditions and reagents of the intended experimental design [14]. This aspect would ensure the selectivity of the assay design for potency determination of Gentamicin Sulfate only without any interference [14]. All pharmaceutical formulations were prepared without the concerned active material (i.e., Gentamicin Sulfate) to assess this parameter.

### 2.3. Establishment of Linearity Curve

To evaluate the validity of the calibration curve, five doses of the standard Gentamicin Sulfate of known potency were used [15]. The range coverage corresponded to aliquots - expressed as µg/mL - of 0.6931 to 3.4657 range (expressed on natural logarithm scale) at a two-fold increment increase. All preparations and dilutions were made in volumetric flasks using buffer No. 3 [16]. The linearity was evaluated by linear regression analysis and correlation between the logarithm of the sample concentration and the inhibition halo diameter and the calculation was conducted using the least-squares method and fit verification by checking the residual plot [17]. Six linearity readings were averaged for each dilution to calculate the standard curve.

### 2.4. Accuracy of the Microbiological Design

The accuracy was determined by adding known amount of Gentamicin Sulfate substance to the samples of the finished product formulation [17, 18]. Accuracy was evaluated by comparing theoretical potency and experimentally determined potency for each level studied at 50, 100 and 150% of the target activity, using linear regression analysis

[19]. In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined [20]. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure [20, 21]. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

### 2.5. Precision of the Microbiological Design

Precision of the method was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability and ruggedness were assessed. Precision was determined through relative standard deviation (RSD). It was also evaluated if precision is associated with concentration through linear regression analysis, by plotting RSD versus concentration. This resulted in the verification that precision is not associated to concentration when the angular coefficient reliability interval includes value zero [22-24].

### 2.6. Robustness of the Microbiological Design

The robustness of the method was determined by analyzing the same sample under a variety of conditions [25]. The factors considered were incubation time, temperature and pH (antibiotic medium no. 1) [17, 26]. Minor and limited deviations or drifts from the standard official assay conditions should not adversely impact the resultant computed potency and it should not show significant shift from expected estimate from that calculated under ideal experimental and laboratory conditions [27]. Based on the compendial requirements, the limits of quantification and detection are not needed for this type of assays [28].

## 3. Results

The inhibition zone microbiological antibiotic assay of Gentamicin Sulfate using balanced two-dose Parallel Line Model (PLM) design was validated in terms of specificity, linearity, accuracy, precision and robustness. The estimated pooled confidence ratio could be estimated between 0.9 and 1.1 which was fairly within the hypothetical criterion window of the claimed target potency of 100%.

### 3.1. Specificity of Microbiological Test Design

The treated placebo (without API Gentamicin Sulfate) preparation under test conditions and processing did not produce any inhibition zone in agar plates after incubation and the microorganism showed homogenous confluent growth throughout the whole plate. This was in contrast to the positive control group where the antibiotic showed a well-defined inhibition zone under similar experimental conditions.

### 3.2. Linearity Curve Analysis

The validation method yielded excellent results for linearity (r = 0.9905). Figure 1 summarizes the results of linearity. The method showed the calibration curve in the studied range, with a correlation coefficient of 0. 9905 and linear equation of:

$$Y = 6.6234X + 15.227 \tag{1}$$

where Y = zone diameter (mm) and X = test solution concentration (µg/mL) expressed as logarithm of the base ten. Upon inclusion of the activity factor (1.077), the constant would be 16.399 and X term would be in I.U./mL. The standard deviations (S.D.) of the ascending doses of Gentamicin Sulfate were 0.305, 0.314, 0.164, 0.342, 0.309, with R-Sq = 98.1% and Standard error of the regression (S) = 0.506885. The Analysis of Variance (ANOVA) for regression, error and total source with Degree of Freedom (DF) of one, three and four showed Sum of Squares (SS) 39.7537, 0.7708 and 40.5245, respectively.

The Mean Square (MS) for regression and error terms were 39.7537 and 0.2569, respectively. The F-calculated was found to be 154.72. The p-value for linearity is equal to 0.001, which is less than the significance level (α) of 0.05. Thus, the result indicated that the association between the inhibition zones and log concentrations is statistically significant. The errors are independent (random) as could be seen in Figure 2. In this normal probability plot, the residuals generally appear to follow a straight line. Table 1 shows the tabulated relation between dose levels of Gentamicin Sulfate and the ranges of zone size, radius and RSD (expressed as %) of diameter for each.
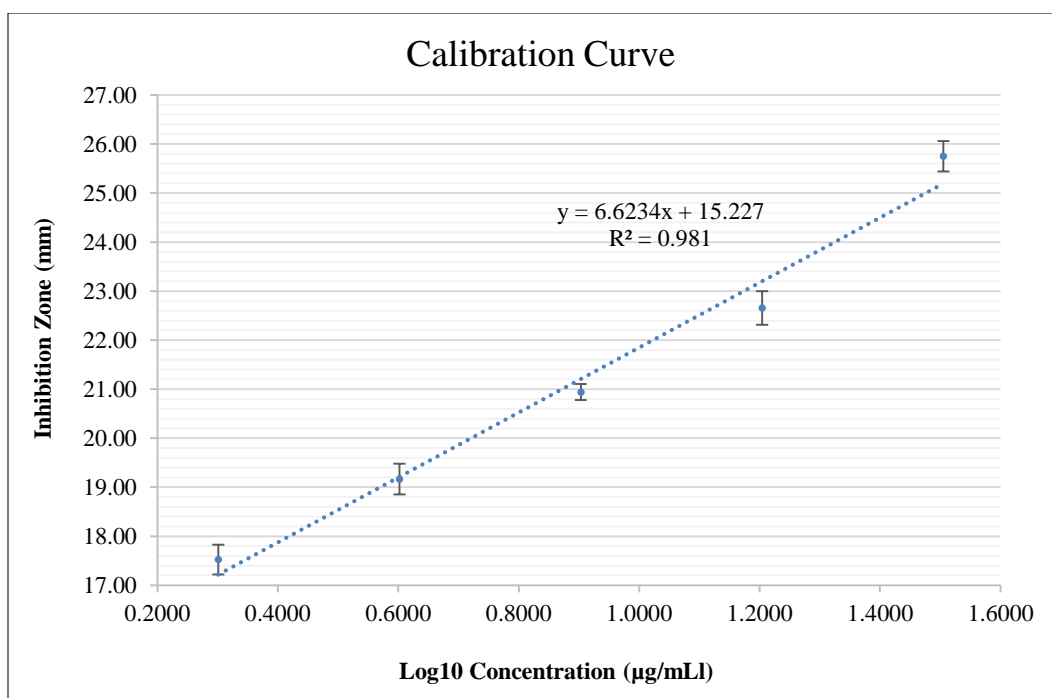
**Figure 1. Calibration curve for Gentamicin linearity assessment covering the range between 0.0002 and 0.0032 g/100 mL w/v (%) showing mean ± S.D**

### 3.3. Accuracy Validation Assessment

The recovery test was performed with three different concentrations and the mean recovery was found to be 100.53% of the target value (Table 2) and RSD of 0.43%, which confirms the ability of the method to accurately determine the concentration of Gentamicin Sulfate in aqueous buffer solution and shows that the results obtained from the bioassay were close to the true concentrations of the samples. The accuracy profile (Figure 3) shows the confinement of the microbiological assay results with 95% confidence intervals (CI) within the acceptance range for the potency determination.

Upon plotting the potencies determined experimentally vs. the theoretical value, a line was obtained. The experimentally obtained values were approximately close to the true values; thus, the line did not shift far from the ideal line, in which the intercept was equal to zero and the slope was equal to one, in turn proving the absence of systemic errors.

### 3.4. Precision and Ruggedness Evaluation of the Design

The precision of the method was determined by repeatability (intra-assay) and intermediate precision (inter-assay). The repeatability (intra-assay) and intermediate precision (inter-assays) were expressed as the relative standard deviation of a series of measurements. The results are shown in Tables 3 and 4. The relative standard deviations (RSD) were well below 5% for all tests conducted for this parameter, thus indicating appropriate intra- and inter-assay precisions.

### 3.5. Robustness of the Potency Determination under Test Conditions Variation

Assessing the test design tolerance to deliberate changes or drifts in the proposed assay conditions showed the robustness of the experimental framework to the deviations in pH of the antibiotic medium (0.2 to 0.6 deviation in pH range), incubation temperature (3 ± 1.5 °C of temperature drift range) and period (time creep of 42 ± 6 hours) of the assay plates as could be seen in Table 5. The computed RSD was found to be acceptable and below 5%.
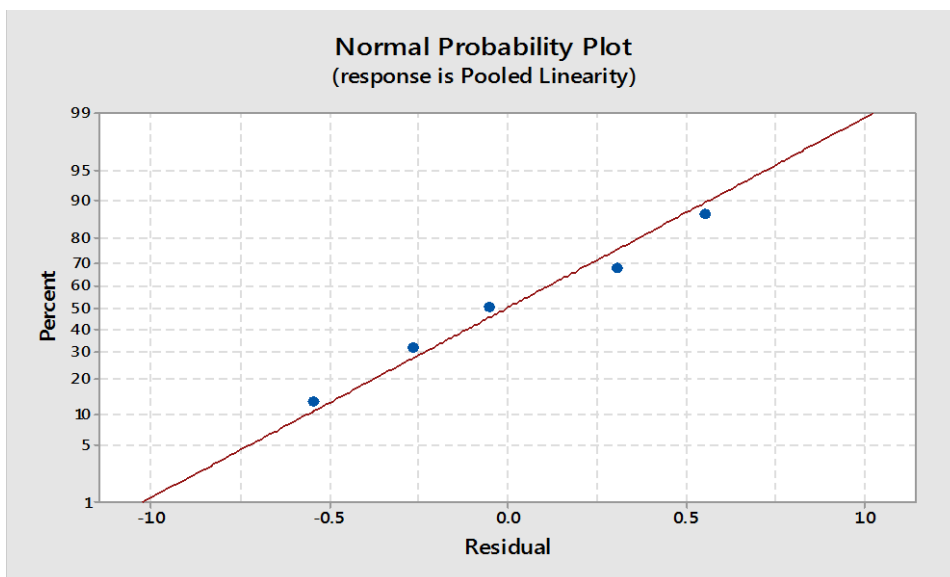
**Figure 2. Q-Q graph for residuals from the linearity curve of Gentamicin Sulfate in PLM**

**Table 1. Inhibition zone diameter (in mm) for Gentamicin Sulfate in USP buffer no. 3 for construction of the linearity curve**

| Expected concentration w/v (%) | Range of computed Inhibition Zone (IZ) area, (mm$^2$) | Average calculated IZ radius*, (mm) | RSD% of measured IZ diameter |
|---|---|---|---|
| 0.0002 | 921.86 – 999.86 | 8.76 | 1.74 |
| 0.0004 | 1110.36 – 1204.41 | 9.58 | 1.64 |
| 0.0008 | 1350.05 - 1406.63 | 10.47 | 0.78 |
| 0.0016 | 1555.28 - 1674.93 | 11.33 | 1.51 |
| 0.0032 | 2006.14 - 2138.44 | 12.88 | 1.20 |

[1] The mean of six reading for each dilution level

* Using calibrated digital caliper with two digits decimal sensitivity from millimeter

**Table 2. Accuracy of PLM microbiological assay of Gentamicin Sulfate in 8×8 rectangular plate**

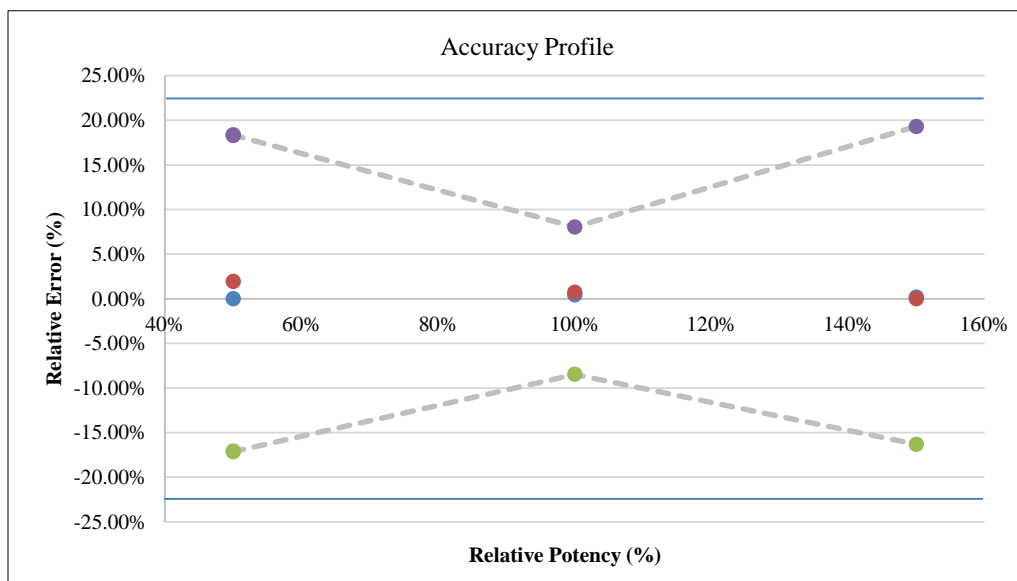| Run | Amount of Gentamicin Sulfate (%) | | | | |
|---|---|---|---|---|---|
| | Theoretical quantity (%) | Mean recovered (%) | Potency (mg/g) | Average recovery (%) | RSD (%) |
| R1 | 50.00 | 50.48 | 0.546 | 100.95 | |
| R2 | 100.00 | 100.56 | 1.088 | 100.56 | 0.43% |
| R3 | 150.00 | 150.12 | 1.624 | 100.08 | |



**Figure 3. Accuracy profile obtained for method of microbiological dosage of Gentamicin Sulfate using 2×2 design in large antibiotic plates. Solid lines represent acceptance limits (-22.5%, 22.5%) around the target value, dashed lines represent 95% tolerance interval reached. When tolerance intervals are confined within specification limits, the assay can be quantified with reasonable accuracy.**
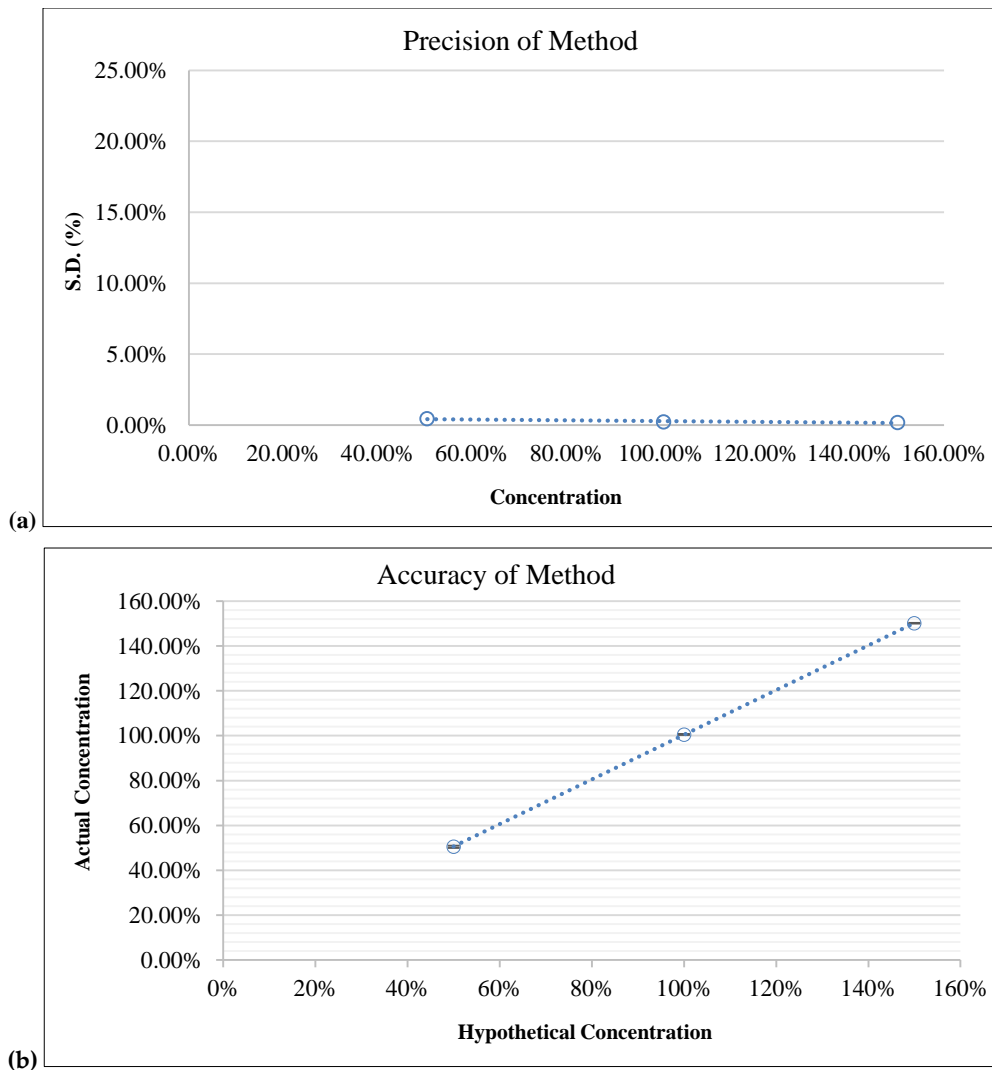
(a)



(b)

**Figure 4. Fixed and relative bias detection and monitoring that showed the angular coefficient, including zero and one terms: (a) Method precision; (b) Method accuracy**

**Table 3. Precision assessment result of PLM microbiological assay of Gentamicin Sulfate**

| Hypothetical target value (%) | Experimental results (%)* | Potency (mg/g) | Intra-assay RSD (%) | Error (%) | Mean potency (%) | Inter-assay RSD (%) |
|---|---|---|---|---|---|---|
| 100.00 (eq. to 1.082 mg/g) | 100.40 | 1.086 | 0.85 | 0.40 | 100.02 (eq. to 1.082 mg/g) | 1.07 |
| | 99.20 | 1.073 | | 0.80 | | |
| | 101.52 | 1.098 | 0.84 | 1.52 | | |
| | 100.30 | 1.085 | | 0.30 | | |
| | 98.43 | 1.065 | 1.32 | 1.57 | | |
| | 100.26 | 1.085 | | 0.26 | | |

\* Three different duplicate repeatability with 16 readings for high and low doses of standard and sample per assay test plate

**Table 4. Between-analyst (ruggedness) result of Gentamicin Sulfate in large assay plates for two-dose PLM**

| Analyst[b] | Practical Potency (%)* | Potency (mg/g) | Intra-assay RSD (%) | Mean group potency (%) | Error (%) | Difference between analysts (%) | Mean overall potency (%) | Inter-assay RSD (%) |
|---|---|---|---|---|---|---|---|---|
| A | 102.50[a] | 1.109 | 1.42 | 101.50 (eq. to 1.098 mg/g) | 2.50 | 0.33% | 101.34 (eq. to 1.096 mg/g) | 1.39 |
| | 100.50 | 1.087 | | | 0.50 | | | |
| B | 99.78 | 1.080 | 1.94 | 101.17 (eq. to 1.095 mg/g) | 0.22 | | | |
| | 102.56 | 1.110 | | | 2.56 | | | |

\* Each experimental group was done in duplicate with 16 readings for high and low doses of standard and sample per assay test plate.

[a] Single outlier value in the unknown low-dose test group was detected, omitted and replaced using USP rule of the replacement of the aberrant values that exceed G critical value.

[b] Intermediate precision for the measurement of the assay reproducibility under analyst variation condition.

**Table 5. Robustness assessment results of Gentamicin Sulfate in large assay plates for two-dose PLM**

| Condition | Practical Potency (%)* | Potency (mg/g) | Mean group potency (%) | Error (%) | Deviation from control (%) | Mean overall potency (%) | RSD (%) |
|---|---|---|---|---|---|---|---|
| Control[b] | 101.08 | 1.094 | 100.08 (eq. to 1.083 mg/g) | 1.08 | N/A | 100.31 (eq. to 1.085 mg/g) | 1.87 |
| | 99.08 | 1.072 | | 0.92 | | | |
| Incubation time | 101.24 | 1.095 | 101.24 (eq. to 1.095 mg/g) | 1.24 | 1.15 | | |
| | 101.24 | 1.095 | | 1.24 | | | |
| Incubation temperature | 97.45[a] | 1.054 | 98.01 (eq. to 1.060 mg/g) | 2.55 | 2.10 | | |
| | 98.56 | 1.066 | | 1.44 | | | |
| Medium pH | 99.92[b] | 1.081 | 101.91 (eq. to 1.103 mg/g) | 0.08 | 1.81 | | |
| | 103.89 | 1.124 | | 3.89 | | | |

[*] Each experimental group was done in duplicate

[a] Single outlier value in the low-dose group was detected, omitted and replaced using USP rule of the replacement of the aberrant values that exceed G critical value

[b] Aberrant value detected in low-dose test was found not representing true outlier due to data condensation and clustering. Thus, decision was made to not rejecting it

### 3.6. Statistical Verification of Assay Validity using Analysis of Variance (ANOVA)

Examination of the assay suitability was conducted statistically using Analysis of Variance (ANOVA) in Table 6. All tests conducted for validation parameters were screened for the validity of the outcome by investigating the pharmacopeial requirements for a two-dose parallel line balanced design of regression and parallelism. The F-calculated for each assay was compared against the tabulated limiting values and was found within the acceptable threshold (>12.56 for regression and <2.83 for parallelism). Thus, all tests for accuracy, precision and robustness were valid to derive the sample potencies of Gentamicin Sulfate. Moreover, the computed probabilities for each experiment were calculated.

## 4. Discussion

The use of an adequate experimental design in relation to the criteria of linearity, precision and accuracy of the analytical results are fundamental requirements for a reliable potency determination test [29]. It is highly advisable to adopt an assay design which, without further effort, gives better results [30]. The number and nature of the samples are the most important factors to be taken into account, in the selection of a design [24, 30]. The 2×2 assay design also known as a symmetrical and balanced assay is simple and effective which employs two doses of standard and two doses of the sample with the same concentration [24, 30]. The microbiological antibiotic assay is a simple, cheap and activity-indicating test for the potency determination of the antimicrobials [15]. However, design suitability and validation should be assessed to ensure the validity of the computed potency from the assay [31]. A prominent focus herein is on Gentamicin Sulfate which is listed in the internationally known reference pharmacopeias as raw material and as a finished pharmaceutical preparation for topical and parenteral administration [3, 6, 16, 32].

This aminoglycoside antibiotic is composed of five main related compounds. The constituents could be differentiated chemically into C1, C1a, C2, C2a, C2b, in addition to multiple minor components by substitution at the 6' carbon (C) of the purpurosamine unit [33-38]. While the analysis criteria for individual compounds may show wide variations in the commercial products that could reach 20% in the range, it would be necessary to use a sort of biological test to estimate the net resultant true activity with this complex mixture of microbiologically active entities [3, 6, 16]. Moreover, Intra-laboratory and inter-laboratory variation mitigation in the microbiological assay might be acquired through the implementation of the international guidelines for the antibiotic assay that include media composition, reagents and assay conditions [39].

### 4.1. Specificity of the Assay Design

The layout of the activity testing procedure must ensure capturing of the intended material potency without misleading estimation of the true activity due to uncontrolled influence of other components present in the test course that could lead to unintentional bias in the assessment of the actual activity of Gentamicin Sulfate [40]. The proposed assay design and conditions showed selectivity toward the response from Gentamicin Sulfate only without any detectable interference from other materials such as reagents, solvents, different other active components or excipients of the pharmaceutical formulation. Specificity is an important criterion to avoid any possible microbiological interference from other unintended factors that would otherwise pertain to the intended active antibiotic material [41]. Generally, these interfering factors include the reagents of the assay or other constituents of the pharmaceutical products either active or inert [42]. Thus, any zone of inhibition in the agar plates could be attributed to Gentamicin Sulfate only [42]. Accordingly, the product without the Active Pharmaceutical Ingredient (API) – called Placebo herein - was used under the exact same assay conditions to exclude the biological interference possibility.

**Table 6. Analysis of variance (ANOVA) of validation group for assessment of the assay design validity**

| Source of Variance | Validation Group | Sum of Squares | Mean Square | Variance Ratio | Calculated Probability |
|---|---|---|---|---|---|
| | | **Accuracy** | | | |
| | 50% | 324.45 | 324.45 | 591.62 | |
| | 100% | 357.59 | 357.59 | 2311.85 | <0.0001 |
| | 150% | 217.12 | 217.12 | 508.40 | |
| | | **Repeatability** | | | |
| | I | 255.28 | 255.28 | 3051.11 | |
| | II | 295.45 | 295.45 | 1962.22 | <0.0001 |
| | III | 221.97 | 221.97 | 974.79 | |
| Regression Squares[a] | | **Ruggedness** | | | |
| | A | 419.78 | 419.78 | 1193.38 | |
| | B | 356.97 | 356.97 | 1561.10 | <0.0001 |
| | | **Robustness** | | | |
| | Control | 260.70 | 260.70 | 1260.59 | |
| | Incubation Time | 574.96 | 574.96 | 1738.60 | |
| | Incubation Temperature | 178.00 | 178.00 | 252.20 | <0.0001 |
| | Medium pH | 63.98 | 63.98 | 450.74 | |
| | | **Accuracy** | | | |
| | 50% | 4.11 | 1.43 | 2.62 | 0.0723 |
| | 100% | 0.53 | 0.18 | 1.13 | 0.3891 |
| | 150% | 1.12 | 0.37 | 0.88 | 0.5040 |
| | | **Repeatability** | | | |
| | I | 0.08 | 0.03 | 0.30 | 0.7170 |
| | II | 1.27 | 0.42 | 2.82 | 0.0572 |
| | III | 0.27 | 0.09 | 0.40 | 0.7080 |
| Parallelism Squares[b] | | **Ruggedness** | | | |
| | A | 1.82 | 0.61 | 1.73 | 0.2019 |
| | B | 0.79 | 0.26 | 1.16 | 0.3796 |
| | | **Robustness** | | | |
| | Control | 1.58 | 0.53 | 2.55 | 0.0784 |
| | Incubation Time | 1.71 | 0.57 | 1.72 | 0.2039 |
| | Incubation Temperature | 0.35 | 0.12 | 0.17 | 0.6603 |
| | Medium pH | 0.39 | 0.13 | 0.92 | 0.4839 |

[a] F-tabulated limiting value >12.56 for d.f. of one

[b] F-tabulated limiting value <2.83 for d.f. of three

## 4.2. Validity of the Linearity Curve

Generally, dose-response relations are not a straight line, but linearity can be achieved through transformation [43]. One of the most commonly used methods for transformations is the logarithmic transformation [44]. The ANOVA and linear regression methods are reasonably robust to mild departures from assumptions regarding constant variance or normality [45]. In many cases, data can be transformed so the transformed response will be sufficiently close to constant variance and normality [44]. To determine whether the association between the response and each term in the model is statistically significant, the p-value was compared for the term to the assigned significance level to assess the null hypothesis.

The null hypothesis is that the term's coefficient is equal to zero, which indicates that there is no association between the term and the response [46]. Usually, a significance level (denoted as α or alpha) of 0.05 works well. A significance level of 0.05 indicates a 5% risk of concluding that an association exists when there is no actual association [46]. The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [47].

It must be realized that linearity will only hold over a certain range and that doses outside this range may give rise to misleading results. Biological concentration-response relationships generally are not linear. The antibiotic potency method allows fitting the data to a straight line by evaluating a narrow concentration range where the results approach linearity. The assay results can be considered valid only if the computed potency is 50%–150% of that assumed in preparing the sample stock solution. When the calculated potency value falls outside 50%–150%, the result for the sample may fall outside the narrow concentration range where linearity has been established. In such a case, adjustment of the assumed potency of the sample would be needed accordingly, and the assay should be repeated to obtain a valid result. It was verified that the methods present linearity when the correlation coefficient (r) is greater or equal to 0.90 and the regression significance is less than 0.01 [22-24].

## 4.3. Accuracy Evaluation of the Assay Design

Accuracy is another criterion in the validation that must be fulfilled. The accuracy was proved by recovery tests performed for the examined experimental designs to determine the agreement between the values found of the analyte and the real value from those analyses [7, 48]. The recovery test was performed with three different concentrations (50%, 100% and 150% of the target value) and the average recovery was computed to be 100.53% of reference substance as could be calculated from Table 2. The methods were considered accurate when the reliability intervals of linear and angular coefficients include, respectively, values of one and zero [24]. The investigated design did not show any fixed or absolute tendency and relative tendency, nor being necessary to employ any kind of correction or adjustment of the results obtained [24]. The method had appropriate accuracy, as could be confirmed by the values calculated for the β-tolerance interval (Figure 3) for each concentration level, which showed a maximum variation of ± 17.8% for the current two-dose symmetrical design [39, 49-51].

Accuracy is represented by the combination of the random (precision) and systematic (trueness) errors, which were considered in the β-tolerance interval calculation. This represents the interval in which β percentage of the future individual results would be expected [39, 50]. According to the trueness parameter, there was no evidence indicating systematic errors in either experimental design [39, 49, 52]. Upon plotting the potencies determined experimentally vs. the theoretical value, a line was obtained. The experimental values were approximate to the true values; thus, the line did not shift away from the ideal line, in which the intercept was equal to zero and the slope was equal to one, in turn proving the absence of systemic errors [39, 52].

## 4.4. Repeatability and Ruggedness Assessment of the Design

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay or ruggedness) which results were expressed as RSD of a series of measurements. In the microbiological assay, the number of replications per dose must be sufficient to ensure the required precision. Furthermore, the assay may be repeated and the results combined statistically to obtain the required precision [7, 15]. The repeatability was studied by determination of the samples in three assays, at the same concentration, under the same experimental conditions. The result obtained shows RSD of acceptable results indicating good intra-assay precision. Inter-assay variability was calculated showing RSD of reasonable value.

## 4.5. Robustness Analysis of the Assay Design

It is defined as the reliability of an analysis with respect to deliberate variations in method parameters [39, 53]. The most important factors of concern in the microbiology laboratory that could influence the analysis comprise pH of the antibiotic medium, incubation time and temperature. The datasets obtained from the conducted experiments showed reasonable stability against variations in the standard assay conditions. Thus, it could be concluded that the assay design would be able to withstand the commonly expected fluctuations in the laboratory experimental conditions for the microbiological assay activities.

## 4.6. Statistical Intervention of the Assay Suitability

Implementation of statistical analysis in the evaluation of the microbiological antibiotic assay is crucial to ensure quality and confidence in the derived potency from the test [54]. It should be noted that doubling the number of the replicates in the treatment groups in each preparation in the balanced assay imposed a significant reduction in the pooled confidence window so that it decreased by 67.58% and reached 95.83% to 104.36% with a range of 8.53% (the minimum acceptance criterion range is 35%). Hence, it is important to control this parameter in the assay based on the main target of the potency determination and its acceptance criteria. For instance, the assay for screening antimicrobial properties would have different requirements and specifications than that for bulk or intermediate manufactured preparations and finished prepared products [54].

The application of the completely randomized design (RCD) in the symmetric PL model is dependent on the fulfillment of the following assumptions [12]. The first assumption is the randomization which would limit variances

that could arise across the assay plates in the inhibition zone experiments. The different treatments have been randomly distributed across rows and column of the assay plate. The second assumption is the normality. The responses to each treatment are normally distributed [54]. However, British pharmacopeia stated that minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are included as could be demonstrated in Figure 5.

The third assumption is homogeneity of variance (Figure 6). The standard deviations of the responses within each treatment group of both standard and unknown preparations don't differ significantly from one another. The fourth assumption is the linearity [54]. The relationship between the logarithm of the dose and the response can be represented by a straight line. The last assumption is the parallelism. For any unknown preparation in the assay, the straight line is parallel to that of the standard as could be observed in ANOVA of Table 6. The regression analysis and deviation of parallelism are mandatory requirements for suitability of the microbiological antibiotic assay design with 2:1 and 4:1 dose ratio as could be demonstrated in several pharmacopeias (e.g., Brazilian, British and Indian) [30].
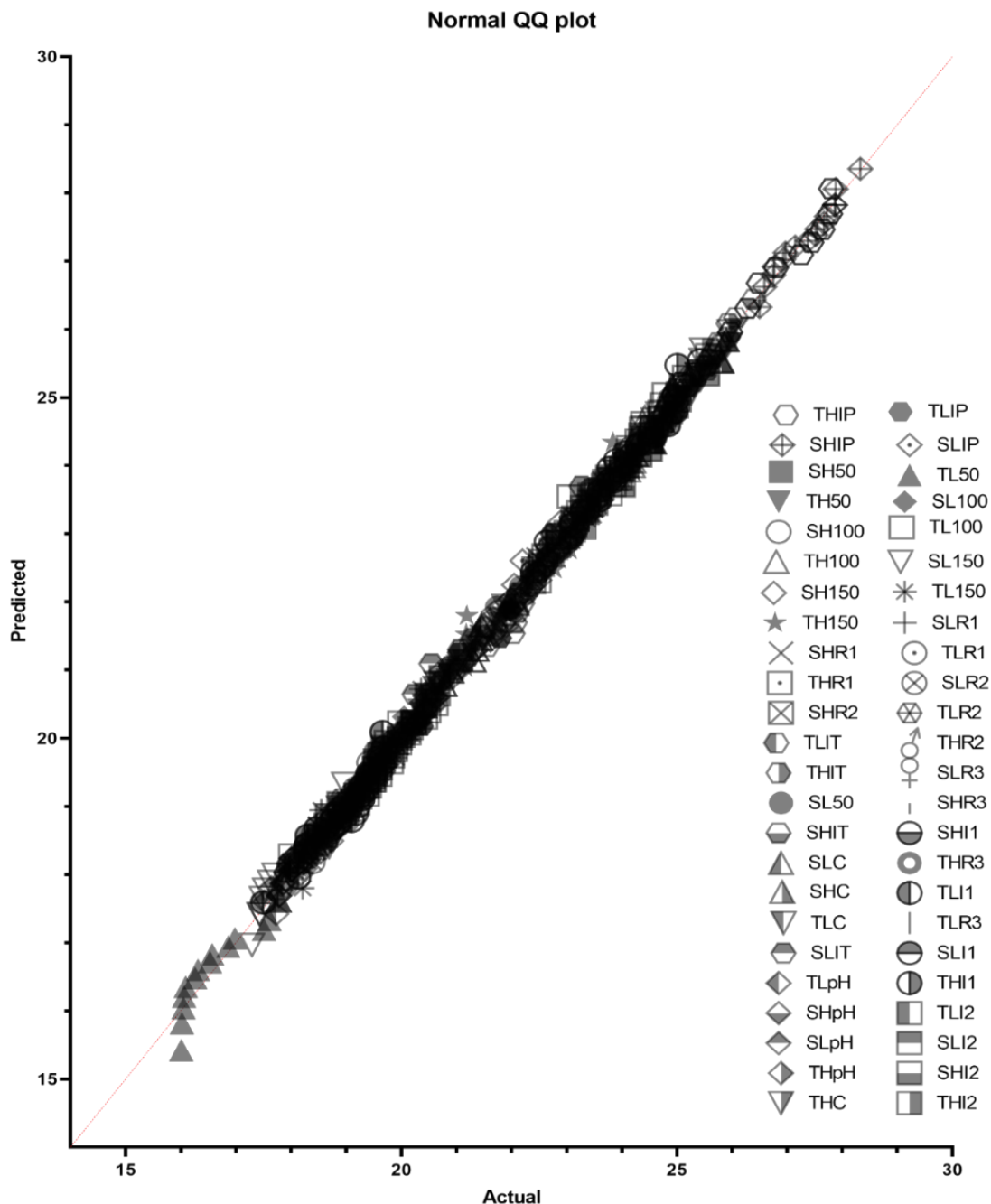


**Figure 5. Q-Q plot for normality of all test groups in the validation study showing the predicted values against the actual data obtained from the experiment (red line is the ideal relationship): S: Standard, T: Test, H: High dose, L: Low dose, R: Repeatability, I: intermediate precision, IP: Incubation Period, IT: Incubation Temperature, 50: 50% accuracy test, 100: 100% accuracy test and 150: 150% accuracy test.**
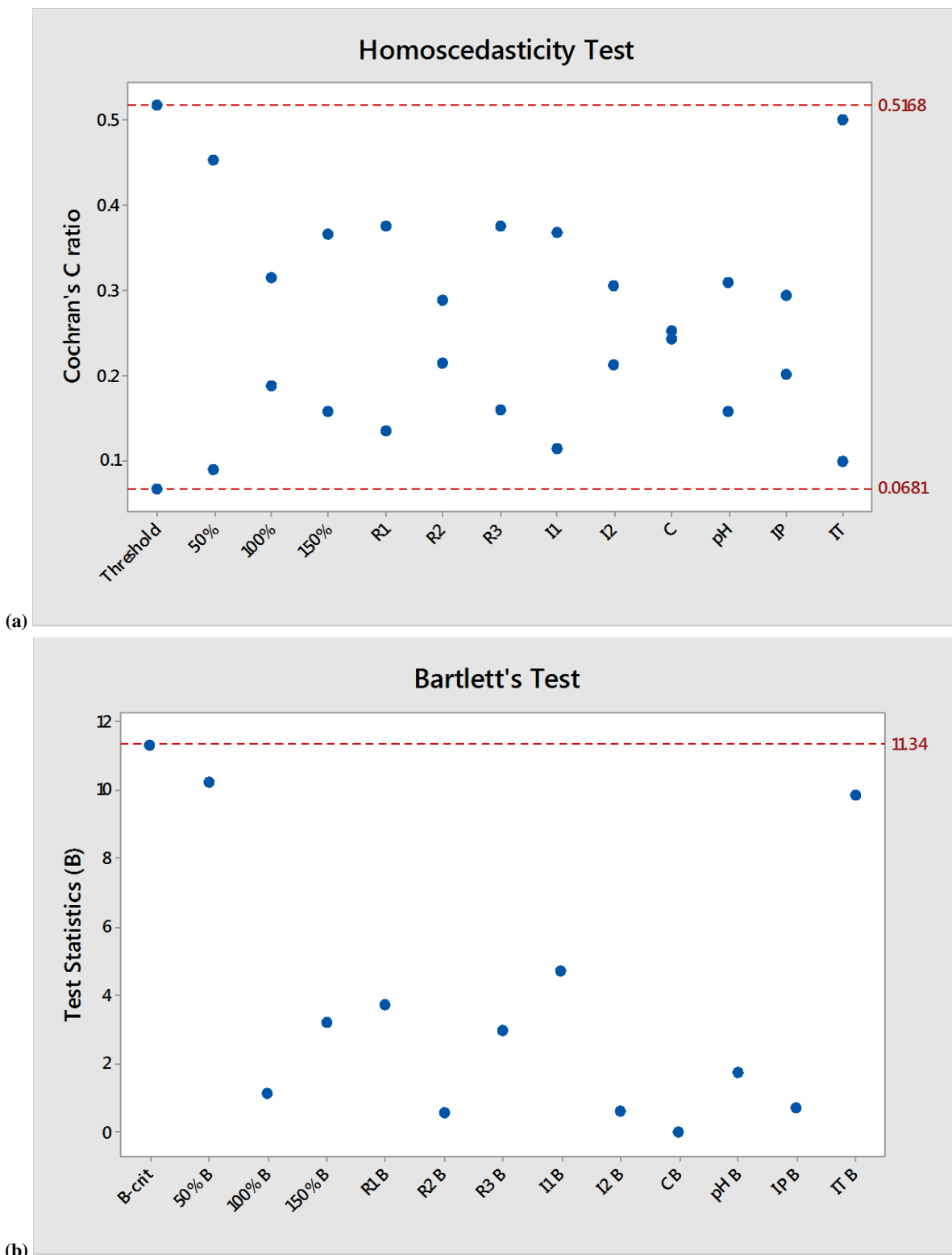
**Figure 6. Homogeneity of variance test showing all experimental groups (R: Repeatability, I: intermediate precision, IP: Incubation Period, IT: Incubation Temperature, 50%: 50% accuracy test, 100%: 100% accuracy test and 150%: 150% accuracy test) within critical values (red dashed line) using: (a) Cochran's test (b) Bartlett's test**

## 5. Conclusion

The microbiological assay is one of the most important analytical techniques that are still in use for several biologically-active compounds especially those that consist naturally of a mixture of several active related components. These types of assays retain simplicity and safety, in addition, to being not expensive. The activity of the antimicrobial compounds should be determined using specific microorganisms which show a measurable response over a predefined linear range against a standard material of the same substance of known potency. Microbiological antibiotic assay of Gentamicin Sulfate - using 2×2 balanced PLM agar diffusion technique in large 30×30 cm large

rectangular (8 rows x 8 columns) antibiotic plates - was assessed using validation parameters of specificity, linearity, accuracy, precision and robustness, in addition to the examination of datasets suitability and assay design validity for potency determinations of this aminoglycoside antimicrobial antibiotic. The examined design showed acceptable results and validation parameters. Thus, it is suitable for the assay of the antibiotic with reasonable confidence. When the confidence range is needed to be more restricted, an assay modification that includes an increase in the number of replicates must be investigated. Recorded assay groups should demonstrate acceptable normality and homogeneity of variance. Moreover, statistical investigation of each experiment dataset could be easily verified for its suitability using ANOVA through a commercial statistical software package. The basic sources of variance were regression and parallelism. All these tests passed the statistical acceptance criteria. Nevertheless, other non-compendial factors that might contribute to the variation could be investigated in another planned future work. This balanced design would be useful for the implementation in the potency determination of Gentamicin Sulfate in both crude forms and in the final finished medical preparation.

## 6. Declarations

### 6.1. Author Contributions

Conceptualization, D.E.E. and E.R.R.; methodology, M.E.E.; software, M.E.E.; validation, E.R.R., D.E.E. and M.E.E.; formal analysis, M.E.E.; investigation, E.R.R.; resources, D.E.E.; data curation, M.E.E.; writing—original draft preparation, M.E.E.; writing—review and editing, E.R.R.; visualization, E.R.R.; supervision, E.R.R.; project administration, D.E.E.; funding acquisition, D.E.E.. All authors have read and agreed to the published version of the manuscript.

### 6.2. Data Availability Statement

The data presented in this study are available in article.

### 6.3. Funding

### 6.4. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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