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Development of High Throughput Rapid Turbidimetric Assay for Potency Determination of Gramicidin

Mostafa Essam Eissa[1]✉ • Engy Refaat Rashed[2] • Dalia Essam Eissa[2]

[1] Independent Researcher, Pharmaceutical and Healthcare Research Facility, Cairo, Egypt, mostafaessameissa@yahoo.com
[2] National Centre for Radiation Research and Technology, Cairo, Egypt, engyrefaat@yahoo.com
[3] Royal Oldham Hospital, Rochdale Rd, Oldham OL1 2JH, United Kingdom, daliaessameissa@yahoo.co.uk

✉ Corresponding Author: mostafaessameissa@yahoo.com

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ABSTRACT

Gramicidin is a polypeptide antibiotic composed of a mixture of antimicrobial compounds. Thus, its antibacterial activity is preferentially assessed using a microbiological assay. The aim of this study is targeting to establish and validate a microbiological potency for Gramicidin with a view to the employment of a simple method with more than two folds output per test run (if compared with symmetrical designs) using 3×1 experimental designs with reasonably statistically acceptable results. The validation criteria of gramicidin turbidimetric assay using the USP method were tested in terms of selectivity, linearity, accuracy, precision and robustness. Moreover, the consistency of the experimental groups was examined in terms of error and difference from the target labelled concentration of 0.25 mg g^{-1} value, in addition to the uncertainty factor. Verification of the assay suitability was evaluated statistically against reference antibiotics of known activity. Calibration of the analytical curve showed a coefficient of correlation (r) = 0.9980 with none of the relative standard deviations (RSD) values greater than three. There was no observable fixed or variable deviation in the absorbance measurement with concentration increment. The accuracy output and profile were evaluated over ranges 50%, 100% and 150% having a maximum RSD of around three with reasonable results, confidence and absence of concentration-related bias. Robustness, precision and suitability verification were evaluated with no outliers and all RSDs below five. The turbidimetric assay design of 3×1 for gramicidin showed acceptable validation parameters and could be used as a substitute design for conventional higher-level parallel line assay models.

INTRODUCTION

Quality control monitoring of the medicinal product potency and activity is a crucial task that must be performed to ensure product safety and efficacy (WHO, 2007; Sardella et al., 2021). Even though many biologically active compounds could be analysed chemically nowadays, bioassay still retains its importance in the analysis of specific drugs such as a complex mixture of related antibiotics as was illustrated by other researchers (Eissa et al., 2021a; Eissa et al., 2021b). Until now, microbiological assay

still possesses a critical role in the evaluation of several antibiotic compounds (Balouiri et al., 2016; Dafale et al., 2016). This kind of test has its advantage over HPLC, UPLC and GC methods in terms of simplicity, low cost and safety from multiple hazardous and toxic chemicals (Dafale et al., 2016). Another important feature – according to Dafale et al. (2016) – is the ability to estimate the biological activity of active products that consist of a composite of several related constituents that are hard to evaluate activity using conventional chemical means.

Gramicidin is a polypeptide antibiotic (Figure 1) that consists of gramicidin A, B, and C, three iontophoretic antibiotics that together make up around 80%, 5%, and 15% of the mixture, also known as gramicidin D (Budavari et al., 1996). The mixture has six different types of gramicidin molecules since each has two isoforms (Kessler et al., 2004). They can be produced from soil bacteria called *Brevibacillus brevis*. Gramicidin constitutes 15 amino acid linear peptides (Kessler et al., 2004). This contrasts with the associated known cyclic peptide gramicidin S. Determination of gramicidin potency is conducted through microbiological assay using the turbidimetric method according to the official monographs from international pharmacopeia (British Pharmacopoeia, 2022; United States Pharmacopeia, 2022). The commonly applied assay designs involve 4×4 or 3×3 Parallel Line Models (PLM) (Hewitt, 2003). This would limit the material batches analysed in a single assay run.

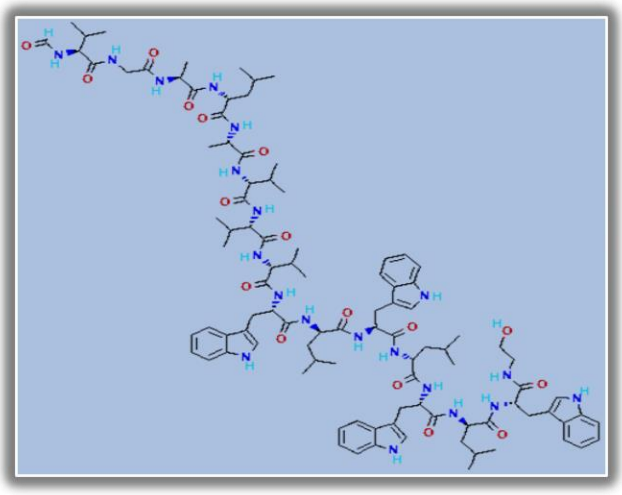

Figure 1. Two-dimensional (2D) structure of the polypeptide antibiotic gramicidin with chemical formula $C_{99}H_{140}N_{20}O_{17}$ (National Library of Medicine, 2007)

The present study herein aimed to investigate an alternative 3×1 simple design with higher throughput but maintaining the quality of the validation criteria with respect to linearity, accuracy, precision and robustness. The availability of this type of design could increase the number of batches throughput that could be tested by more than 300% from the original test design.

MATERIALS AND METHODS

Chemicals

The gramicidin standard (assigned with a potency of gramicidin 0.6019 mol kg^{-1} with batch number $1.31E^{+09}$ was obtained from a local broker, pharmaceutical dosage forms from the market containing gramicidin were obtained commercially from the market retail and it was claimed to have 5 mg 20 g^{-1} of the Active Pharmaceutical Ingredient (API) in medicinal product unit (Eissa et al., 2021a). All reagents used were of analytical reagent grade and were purchased from Oxoid, Merck and Fluka (Oppe et al., 2018).

Microorganisms and Inoculum

The cultures of *Enterococcus hirae* NCTC 13383 (Culture Collections, 2007) were cultivated from a freshly grown slant in antibiotic medium No. 3 – which would also be used in the assay in the oscillating-rack water bath - and incubated at 36.8±0.7°C for 17±1 hours (British Pharmacopoeia, 2022; United States Pharmacopeia, 2022). The stock microbial suspension was prepared at the end of the incubation time by re-suspending the solution and making the appropriate absorbance adjustments with antibiotic medium no. 3. The absorbance was adjusted for inoculum at 2.398 AU measured at wavelength $5.3E^{-7}$ m using a qualified spectrophotometer and about one cm diameter test tube of absorption cells against plain medium as blank (Francisco et al., 2014; Christ et al., 2015).

Reference and Samples Preparation

The reference and the raw material samples solutions were prepared using an amount of powder equivalent to 100 mg of gramicidin that was transferred to a 100 mL volumetric flask with dehydrated Ethanol followed by making up to the final volume with this solvent (1000 μg mL^{-1}). For a topical pharmaceutical product, an amount of about 2 g was transferred to the 100 mL volumetric flask and made up to the final volume with homogenization in absolute Ethyl Alcohol. Final aliquots dilutions range in the diluent were between 1:80 and 1:16 v/v using five equally separated increment levels so that the assay doses - expressed as ln values of (ng L^{-1}) - were 3.219, 3.912, 4.317, 4.605 and 4.828.

Calculations

To calculate the activity (potency) of gramicidin in raw material and pharmaceutical preparation a standard equation was adopted. The assay was statistically calculated by the 3×1 model and by means of regression analysis and verified using analysis of variance (ANOVA) (William, 2003; Eissa et al., 2021c; Eissa et al., 2021d).

Method Validation

The method was validated by determination of linearity, precision, accuracy, robustness and specificity. According to the ICH and the United States Pharmacopoeia (Ermer & Miller, 2006; Oppe et al., 2018), the limits of detection and quantification are not required for this category of assay.

Linearity

The calibration curve was obtained with five doses of the working standard. The linearity was evaluated by linear regression analysis, which was calculated by the least-squares regression method. Five readings were performed (Eissa et al., 2021a).

Precision

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability was evaluated by assaying three samples at the same concentration and on the same day. The intermediate precision was verified by comparing the assays of two different analysts. The precision is calculated by the relative standard deviation (RSD) (Oppe et al., 2018).

Accuracy

The accuracy measurement range was assessed at 50%, 100% and 150% from the target concentration value. This was determined by adding a known amount of the Active Pharmaceutical Ingredients (API) in the samples to yield the hypothetical potency required (Eissa et al., 2021c). The accuracy determinations were evaluated with this concentration range.

Robustness

The experimental framework for the potency determination under the test conditions variation assessing the test design tolerance to deliberate changes or drifts in the proposed assay conditions to show the robustness of the experimental layout to a small deviation in pH of the antibiotic medium (0.4±0.2 deviations in pH range), incubation temperature (37±1°C of temperature drift range) and period (time variation of 210±30 minutes) of the tube assay conditions.

Specificity

The specificity was determined by measurement in presence of the active compound and blank. The blank samples were processed exactly as that containing the active antimicrobial component to examine the possible interference from other assay reagents and chemicals. This should ensure that and change in turbidity could be attributed only to gramicidin.

RESULTS AND DISCUSSION

The calibration curve for gramicidin was constructed by plotting the log (to base ten) of concentration (ng mL^{-1}) versus absorbance (AU) following a similar approach as in previous studies (Figure 2) (Zuluaga et al., 2009; Dafale et al., 2015). The corresponding mean absorbance for reference solutions was 0.163 AU (RSD% = 0.76) for the lowest dose (28.33 ng mL^{-1}), 0.154 AU (RSD% = 1.04) for the next dose (56.65 ng mL^{-1}), 0.146 AU (RSD% = 0.64) for

the middle dose (113.30 ng mL^{-1}), 0.143 AU (RSD% = 0.76) for the fourth dose (141.3 ng mL^{-1}) and 0.139 AU (RSD% = 0.70) for highest dose (169.95 ng mL^{-1}) as could be seen in Table 1. The line fit plot showed the agreement between the actual analytical curve points and the predicted values. Regression investigation showed that there is no sign of a fixed or variable trend in the residual error with concentration (Eissa et al., 2021a, Eissa et al., 2021d). Moreover, the sample points cannot be excluded from the normality assumption as they followed a normal probability plot with good regression and the correlation coefficient is 0.953 (Eissa et al., 2021b, Eissa et al., 2021c). At P = 0.05, the critical value for N = 5 is 0.8786 which is fairly below the calculated value. The variability was low with no observable pattern and RSD% below five. The regression line analysis is shown in detail in Table 2 with a good coefficient of determination and minimum error. ANOVA test showed $F_{actual} > F_{significance}$ (Hewitt, 2012; Nunes Salgado & Gomes Tozo, 2007). The calibration equation could be expressed as the following:

$$y = -0.0303x + 0.2077 \quad (1)$$

Where: y is the absorbance (AU) and x is the logarithmic transformation of the gramicidin concentration in ng mL^{-1} with R^2 = 0.9961. The lower and upper 95% confidence for the coefficients of the regression formula intercept and slope were ±0.006814231 and ±0.003495249, respectively. Statistically, there is no significant difference between the predicted and actual residuals at P < 0.05 when a two-tailed paired t-test was used (Table 3).

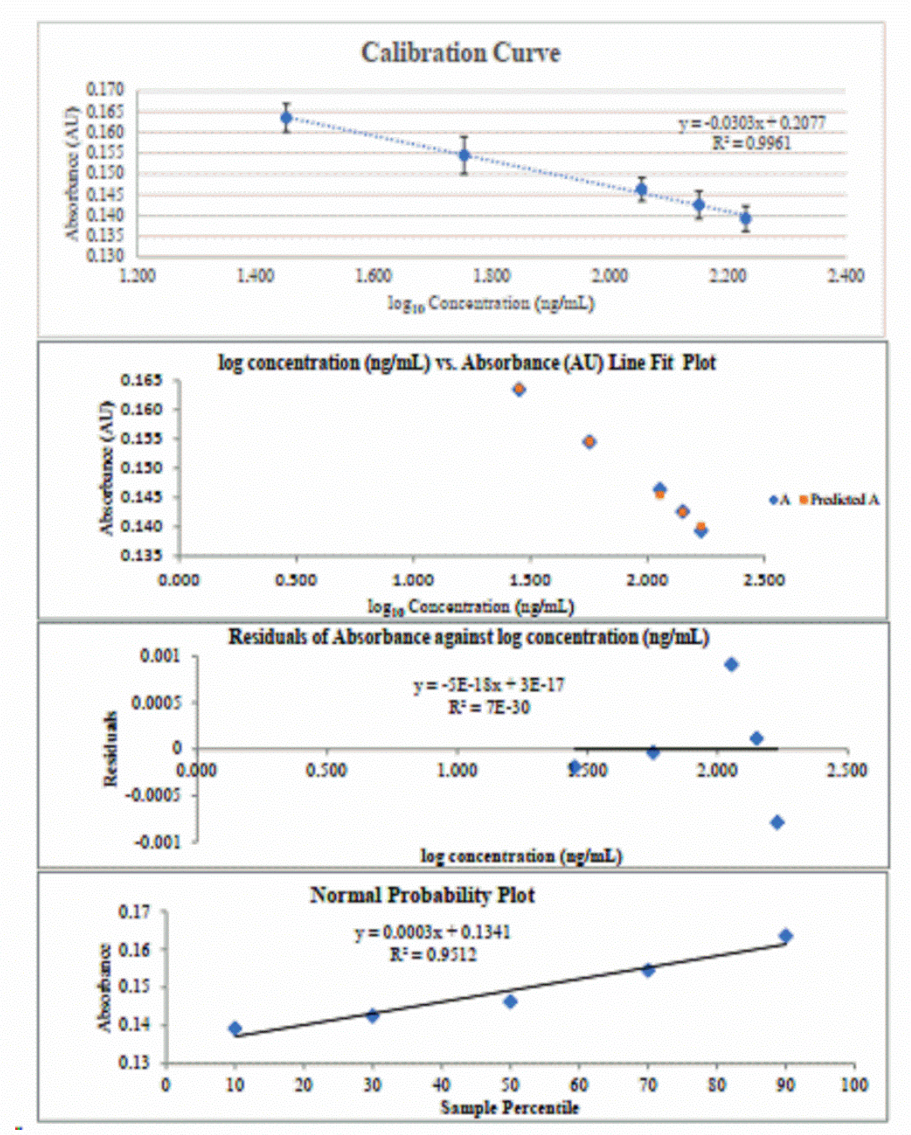

Figure 2. Analysis of linearity curve of gramicidin in turbidimetric assay with the adjusted overall absorbances of high dose (H) at 0.137 and low dose at 0.161 for range 0.024 for five observations (concentrations) levels. The correlation between absorbance and log10 (concentration) is -0.9980.

Table 1. Descriptive analysis of the absorbance readings data for six readings of gramicidin at five concentration levels

Mean Absorbance (AU)*	SD	RSD%	Variance	Combined SD	Combined RSD
0.164	0.0033	%2.04	8.93453E-06		
0.155	0.0045	%2.91	9.91826E-06		
0.146	0.0028	%1.91	6.53651E-06	0.003	%2.30
0.143	0.0033	%2.31	9.0683E-06		
0.139	0.0030	%2.17	8.19301E-06		

Note: * Average of five measurements

Table 2. Statistical analysis for the validity of the linearity curve of gramicidin in turbidimetric assay

Regression Statistics summary output					
Multiple R Correlation	0.99804				
R Square	0.996083				
Adjusted R Square	0.994778				
Standard Error	0.000707				
ANOVA*	**df¥**	**SS€**	**MS£**	**Significance F**	
Regression	1	0.000381	0.000381	0.000104	
Residual	3	1.5E-06	4.99E-07	**F**	
Total	4	0.000383		762.9703702	
Curve Parameter	**Coefficients**	**Standard Error**	**t Stat**	**Lower 95%**	**Upper 95%**
Intercept	0.207713	0.002141	97.00806	0.200899	0.214527
log conc.	-0.03034	0.001098	-27.6219	-0.03383	-0.02684

Note: *Analysis of Variance; €Sum of Square; £Mean Square; ¥Degree of Freedom.

Table 3. Residual and probability outputs for the calibration curve of gramicidin using five concentrations.

Observation	Predicted Absorbance	Residuals*	Standard Residuals	Percentile	Absorbance
1	0.163659	-0.00019	-0.31653	10	0.139263
2	0.154526	-3.8E-05	-0.06175	30	0.142567
3	0.145394	0.000908	1.48312	50	0.146302
4	0.142454	0.000113	0.184469	70	0.154489
5	0.140052	-0.00079	-1.28931	90	0.163465

Note: * The discrepancy between expected results of $\hat{Y}$ (the dependent variable) and actual values of y is the residual for each observation. Relative to projected and actual y values and according to equation 1, residual $(r_i) = y_i - \hat{Y}_i = y_i - (-0.0303x + 0.2077)$

Table 4. Evaluation of the accuracy of the turbidimetric analysis of gramicidin using 3+1 assay design

Group€	Theoretical Potency (mg/g)	Recovered Potency (mg/g)	Recovery (%)	Acceptance Range (90–130%)	%95 CI Range	Maximum G_1* (P=0.02)	SD§	RSD (%)¥
AR1	0.125	0.125	99.82	0.050	0.039	0.646	0.0054	2.95
AR2	0.250	0.251	100.54	0.100	0.087	0.619	0.0044	2.66
AR3	0.375	0.368	98.18	0.150	0.106	0.619	0.0048	3.02
Combined SD§		0.0049	**Average Absorbance (AU)**		0.170	**Combined RSD¥**		2.89

Note: * USP outlier detection: $G_1 = Z_2-Z_1 / Z_n-Z_1$, where Z is the AU reading and the subscript number is the order ascending and descending based on the magnitude The limiting Value of $G_1 = 0.846$ ¥ Relative Standard Deviation § Standard Deviation € Accuracy Result

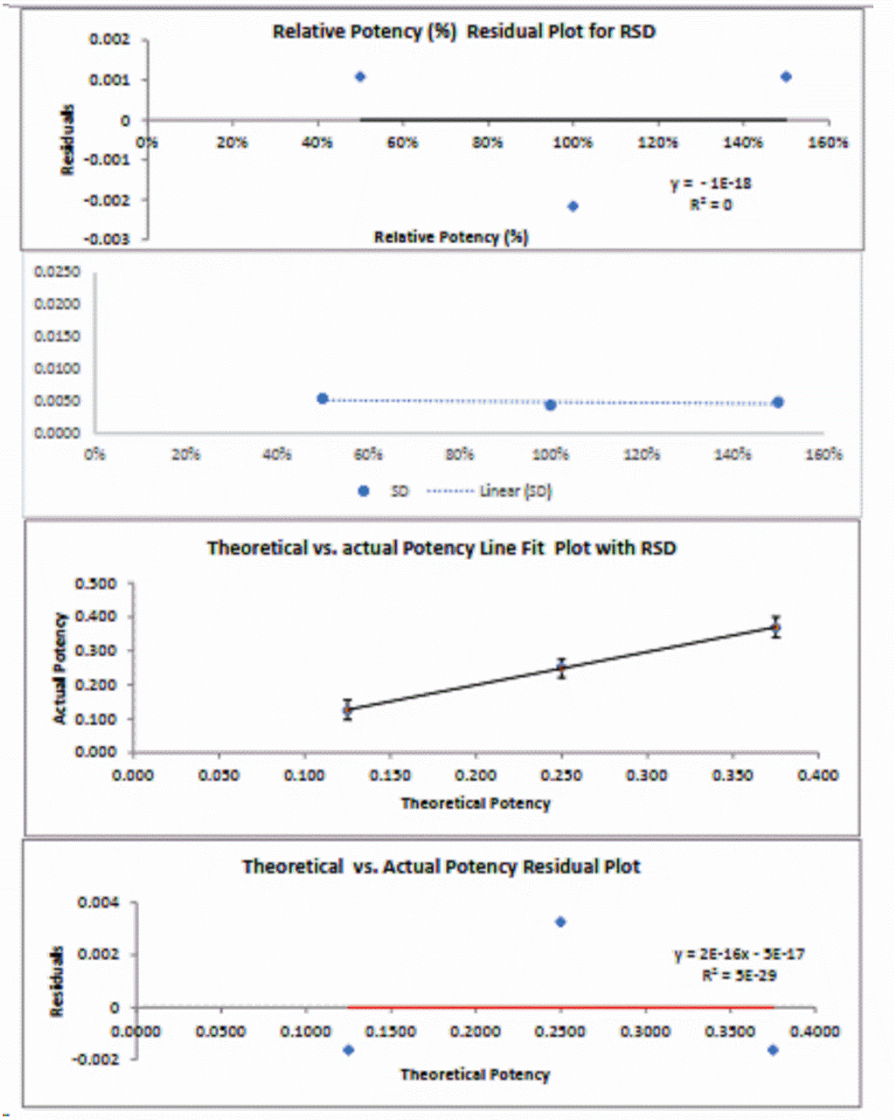

Figure 3. Accuracy profile analysis to assess the presence of signs of fixed and relative bias over the range 50% to 150% from the target potency

Table 5. Consistency and validity verification check analysis for the overall experimental groups and pairwise comparison study between the reference product potency and the 3×1 design for the turbidimetric assay of gramicidin powder for 13 separate tests at 95% confidence interval (CI)

Group	Error from Target (%)	Difference from Theoretical Value (%)	Uncertainty (%)
Control	1.13	1.12	0.011
pH	0.89	0.89	0.009
Incubation Time	0.54	0.54	0.005
Incubation Temperature	2.60	2.57	0.025
AR1	0.18	0.18	0.002
AR2	0.54	0.54	0.005
AR3	1.82	1.84	0.019
RA A	2.16	2.14	0.021
RA B	1.17	1.18	0.012
RP A	1.37	1.36	0.014
RP B	1.61	1.60	0.016
RP C	0.11	0.11	0.001
Lower Extreme G_2	0.199	0.201	0.202
Upper Extreme G_2	0.322	0.306	0.289

Gramicidin Sample Code	Comparison Groups of Gramicidin Assay Designs¥	
	3×1 Design Potency	**Manufacturer Reference Potency**
G_{549}	1066	1081
G_{869}	1029	1066
G_{200}	1093	1072
G_{382}	1053	1057
G_{568}	1086	1072
G_{549r}	1128	1081
G_{145}	1179	1093
G_{145s}	1003	1093
G_{506}	1192	1055
G_{264}	903	1038
G_{612}	1202	1051
G_{145r}	1109	1093
G_{248}	1030	1057

t-Test: Paired Two Sample for Means*		
Mean	1082.54	1069.92
Pearson Correlation	0.2499	
Hypothesized Mean Difference	0	
df	12	
t Stat§	0.5618	
P(T<=t) one-tail	0.2923	
t Critical one-tail	1.7823	
P(T<=t) two-tail	0.5846	
t Critical two-tail	2.1788	

Note: * Significance level (α) = 0.05

§ For null hypothesis of no difference t_{Stat} should be lower than $t_{critical}$

¥ Potency of not less than 900 µg of gramicidin per mg, calculated on the dried basis according to USP and BP G_2 (P = 0.02) threshold value of 0.643, for n = 8 - 13

Accuracy determinations were estimated at 50%, 100% and 150% of the target concentration of the antibiotic (Nunes Salgado & Gomes Tozo, 2007). None of the groups showed significant outliers as the maximum G_1 observed in all treatments was lower than the critical value limit (Eissa et al., 2021a; Eissa et al., 2021d; United States Pharmacopeia, 2022). In addition, the 95% confidence interval (CI) ranges were narrower than the acceptance criteria threshold (Solano et al., 2011). None of the RSD% exceeded 5%. The target and actually recovered potencies can be found in Table 4.

Statistical analysis for the regression statistics of the potency results of the three levels of the observations from the accuracy results showed that the multiple regression (R) value is 0.999732, R^2 = 0.999463, adjusted R^2 = 0.998926 with standard deviation (SD) of ±0.003989. ANOVA examination of the accuracy profile showed the value of F_{test} (1861.987) > $F_{significance}$ (0.014751) with a degree of freedom (df), sum of squares (SS) and mean squares (MS) of unity, 0.029622 and 0.029622, respectively. SS and MS of residual with df of one were estimated to be 1.59E-05 and 1.59E-05, respectively. Total SS with a degree of freedom of two was found to be 0.029638. The curve parameters of intercept and slope were analysed for fixed and variable bias with no statistical evidence that could be observed (Loureno et al., 2007). The intercept coefficient with 95% upper and lower bounds of 0.082114 and -0.07272, respectively. The standard error was calculated as 0.006093, t Stat of 0.771262 and P-value of 0.58176. The slope coefficient was calculated as 0.973607, standard error of 0.022563, t stat of 43.15074, P-value 0.014751 and the 95% upper and lower bounds were 1.260296 and 0.686918, respectively. Thus, the intercept and slope factors embrace one and zero values i.e., 0.004699±0.077417 and 0.973607±0.286689, respectively. These findings are illustrated in Figure 3 which is supported by SD, RSD and theoretical vs. actual plots to show the absence of significant bias in error with the concentration range investigated.

Investigations of the precision and robustness criteria were demonstrated in Table 5 with the outcome in the same line with as previous works (Eissa et al., 2021c). The deliberate minor fluctuations in the selected experimental conditions i.e., pH, incubation time and temperature showed acceptable average recovery (101.02% (RSD% 1.27) = 0.251 mg g^{-1} ± SD 0.00545) with all RSD% values below five. The repeatability and intermediate precision groups yielded statistically valid results with mean recovery of 100.82%, RSD% 1.33 which is equivalent to 0.252 mg g^{-1} ± SD 0.00327.

Table 5 consists of two sections for verification of the suitability of the 3×1 assay design. The first part showed the measurement of the consistency among all experimental groups by measuring percentage error, difference and certainty with reference to the theoretical target value of 100%. There was no aberrant result when using either the USP G test for outliers for n = 8 - 13 at P = 0.02 or robust regression and outlier removal (ROUT) at Q = 10.0% test which is based on the false discovery rate (FDR) (Motulsky, 2015; United States Pharmacopeia, 2022). Furthermore, these groups demonstrated homogeneity of the distribution indicated by showing signs of normality by both the Kolmogorov-Smirnov (KS) normality test and the Shapiro-Wilk normality test at α = 0.05. Thus, variation and error factors throughout the whole experiment did not demonstrate any evidence of abnormality in the consistency among the test groups.

The second aspect of verifying the assay design validity is the comparison of the gramicidin of known reference potency with that generated from the established turbidimetric design using paired t-test for a series of antibiotic materials from a well-known manufacturer source with reference potency (Table 5). The two-tailed parametric test for the customarily distributed group columns showed a significant correlation between test and reference groups without statistically substantial difference where t-actual < t-critical (Vieira et al., 2014; Martins et al., 2020). Hence,

the potency results using an alternative design are comparable with that of the reference control group.

CONCLUSION

The turbidimetric potency determination of the 3x1 design for gramicidin antibiotic showed acceptable validation parameters in terms of specificity, accuracy, precision and robustness. The proposed tube assay could be used as an alternative for the conventional 3×3 or 4×4 assay methods with comparable results that are statistically not significantly different with relatively and remarkably higher output batch analysis per assay run. The assay method is fast, simple, effective and safe without expensive instruments and no significant use of hazardous chemicals or reagents was encountered.

Compliance with Ethical Standards

Authors' Contributions

MEE: Performed laboratory experiments and managed statistical analysis.

ERD: Wrote the first draft of the manuscript.

DEE: Designed the study. Carried out the field study.

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article.

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