Analytical Agreement Between NM BAPTA and Arsenazo III Methods for Calcium Measurement: A Passing-Bablok Regression and Bland-Altman Analysis

Dr. Supraja S.

Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Puducherry, India

Guide: Dr. TMJ Santhoshakumari

ABSTRACT

Background: Accurate quantification of intracellular free calcium (Ca^{2+}) is critical in biochemical and clinical diagnostics. NM BAPTA (N-methyl-O,O′-bis(2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid) and Arsenazo III are two widely employed spectrophotometric methods, yet their analytical agreement has not been rigorously evaluated using non-parametric regression techniques.

Objective: To evaluate the analytical agreement between NM BAPTA and Arsenazo III for calcium measurement using Passing-Bablok regression, Bland-Altman analysis, and supporting statistical methods in a cohort of 100 paired samples.

Methods: One hundred paired calcium measurements (range 40–250 nM) were obtained simultaneously by both methods. Passing-Bablok regression was performed as the primary method comparison technique. Supporting analyses included Bland-Altman limits of agreement, Pearson and Spearman correlation, paired t-test, Wilcoxon signed-rank test, CUSUM linearity testing, and Shapiro-Wilk normality assessment.

Results: Passing-Bablok regression yielded a slope of 1.0722 (95% CI: 1.0523–1.0963) and an intercept of −6.1555 nM (95% CI: −8.5944 to −3.5423 nM). Both confidence intervals excluded their respective null-hypothesis values (slope = 1.0 and intercept = 0), indicating statistically significant proportional and constant systematic errors. Pearson r = 0.9774 ($p < 0.001$) and Bland-

Altman analysis revealed a mean bias of 2.64 nM with limits of agreement of −9.79 to 15.06 nM. CUSUM testing confirmed linearity across the measurement range.

Conclusion: Significant proportional and constant systematic differences exist between NM BAPTA and Arsenazo III for calcium measurement. Although the two methods are highly correlated, direct interchangeability without mathematical correction is not recommended. The Arsenazo III method overestimates calcium by approximately 7.2% relative to NM BAPTA, with an additional negative offset at low concentrations.

Keywords: *Passing-Bablok regression; method comparison; NM BAPTA; Arsenazo III; calcium measurement; Bland-Altman analysis; analytical agreement; CLSI EP09*

1. INTRODUCTION

Calcium (Ca^{2+}) is one of the most tightly regulated ions in mammalian physiology, serving as a ubiquitous second messenger that controls processes ranging from muscle contraction and neurotransmitter release to apoptosis and gene expression. The free intracellular calcium concentration ($[Ca^{2+}]_i$) in resting cells is maintained at approximately 50–200 nanomolar (nM), orders of magnitude lower than the extracellular concentration of approximately 1.2 mmol/L. Perturbations in $[Ca^{2+}]_i$ homeostasis have been implicated in a broad spectrum of pathological states including cardiac arrhythmias, neurodegeneration, diabetes mellitus, and malignancy (Berridge et al., 2000; Clapham, 2007).

The precise and reproducible measurement of calcium, particularly at nanomolar concentrations, therefore carries significant diagnostic and research importance. Two analytical approaches are in widespread use in biochemistry laboratories: fluorescent chelation-based methods employing compounds such as NM BAPTA (N-methyl-O,O′-bis(2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid) and metallochromic indicator dyes such as Arsenazo III (2,2′-[1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo]-bisbenzenearsonic acid). NM BAPTA exploits the large spectral shift of its calcium-bound form, affording high sensitivity and selectivity. Arsenazo III, introduced in the 1970s, undergoes a bathochromic absorbance shift on chelation with divalent cations and remains in routine use due to its simplicity and low cost.

Despite their parallel deployment in clinical and research contexts, formal evaluation of the analytical agreement between these two methods using modern method-comparison statistics is lacking. The Passing-Bablok regression procedure, a non-parametric rank-based approach, is regarded as the reference standard for method-comparison studies in laboratory medicine (Passing & Bablok, 1983; CLSI EP09-A3, 2013). Unlike ordinary least-squares (OLS) regression, it assigns measurement error symmetrically to both methods and is robust to non-normality and outliers. Complementary to this, Bland-Altman analysis quantifies clinical interchangeability through mean bias and limits of agreement (Bland & Altman, 1986).

The present study was designed to rigorously evaluate the analytical agreement between NM BAPTA and Arsenazo III for calcium quantification across a representative physiological and pathological concentration range using a comprehensive statistical framework anchored by Passing-Bablok regression.

2. REVIEW OF LITERATURE

2.1 Calcium Measurement Methods

The history of biochemical calcium measurement spans more than six decades. Early flame photometric and atomic absorption spectrometric methods were succeeded by colorimetric indicator assays that permitted high-throughput laboratory analysis. Among these, Arsenazo III was introduced by Michaylova and Ilkova (1971) as a sensitive reagent for the detection of divalent cations. In an acidified environment, the dye undergoes a red-to-blue colour shift upon chelating calcium, with a characteristic absorbance peak at 650 nm. Its ability to discriminate calcium from magnesium through pH manipulation has been extensively exploited in clinical chemistry platforms (Moorehead & Biggs, 1974; Connerty & Briggs, 1966).

Fluorescent chelators for intracellular calcium measurement were introduced by Tsien and colleagues with the development of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) and its fluorescent derivatives (Tsien, 1980). NM BAPTA, the N-methylated analogue, retains high calcium selectivity ($K^d \sim 100–150$ nM) while exhibiting a large ratiometric or absorbance-based response, making it suitable for both fluorimetric and spectrophotometric applications (Harrison & Bers, 1987). Its dissociation constant closely matches the resting $[Ca^{2+}]_i$, providing optimal sensitivity in the physiological nanomolar range.

2.2 Method Comparison in Laboratory Medicine

The evaluation of new or alternative analytical methods against established reference procedures is a cornerstone of clinical chemistry quality assurance. Recognised international standards for method-comparison studies include CLSI guideline EP09-A3 (Clinical and Laboratory Standards Institute, 2013) and ISO 5725-2 (International Organization for Standardization, 1994). These guidelines specify minimum sample requirements, concentration range coverage, analytical replication, and the statistical methods appropriate for assessing proportional and constant bias.

Ordinary least-squares (OLS) regression has historically been applied to method-comparison data, but it rests on the assumption that the independent variable (typically the reference method) is measured without error. This assumption is rarely met in practice and leads to systematic underestimation of the slope (regression dilution bias), making OLS unsuitable as the primary tool for method comparison (Linnet, 1990). In response, Passing and Bablok (1983) developed a non-parametric, rank-based regression procedure that estimates slope as the median of all pairwise slopes between data points, is invariant to which method is designated the dependent variable, and is robust to both outliers and non-normality.

2.3 Passing-Bablok Regression

The Passing-Bablok (PB) procedure estimates the regression slope b as the shifted median of all pairwise slopes $S_{ij} = (y_j - y_i)/(x_j - x_i)$ for $i \neq j$, where slopes equal to -1 are excluded by convention to avoid singularity. The intercept a is then estimated as the median of $(y_i - b \cdot x_i)$ residuals. Confidence intervals are constructed non-parametrically from the ordered slope distribution using a normal approximation for sample sizes $n \geq 10$ (Passing & Bablok, 1983).

The test of proportional error (H_0: slope = 1.0) and constant error (H_0: intercept = 0.0) is based on whether the respective 95% confidence intervals include the null value. A slope significantly different from 1.0 indicates that one method reads systematically higher or lower at greater analyte concentrations (proportional bias), while an intercept significantly different from zero indicates a fixed offset across all concentrations (constant bias). Linearity of the regression model is verified using the CUSUM test, which plots the cumulative sum of signs of residuals ordered by x and compares the trajectory against critical limits at $\pm 1.36\sqrt{n}$ (Passing & Bablok, 1988).

Independent validation of the PB approach against simulation-based methods and clinical datasets has confirmed its superior performance relative to OLS and Deming regression under

conditions of non-normality and heteroscedasticity (Linnet, 1993; Bilić-Zulle, 2011; Martin, 2000). Its adoption in CLSI EP09-A3 has further cemented its status as the recommended technique.

2.4 Bland-Altman Limits of Agreement

While correlation coefficients and regression analyses assess the linear relationship between methods, they do not directly quantify clinical agreement. Bland and Altman (1986) proposed the limits-of-agreement (LoA) method as a clinically interpretable complement. A Bland-Altman plot graphs per-sample differences ($y - x$) against the mean [$(x+y)/2$], and the LoA are defined as the mean bias ± 1.96 standard deviations of differences. Approximately 95% of future differences between the two methods are expected to fall within these limits, assuming normally distributed differences. Clinical acceptability is judged by comparing the LoA against a predefined total allowable error specification.

2.5 Comparative Studies of Calcium Methods

Several previous investigations have addressed the performance characteristics of Arsenazo III. Moorehead and Biggs (1974) reported excellent linearity and precision for Arsenazo III across the range 1.5–3.5 mmol/L in serum. Periasamy and Bhatt (1998) demonstrated high selectivity of Arsenazo III for calcium over magnesium when performed at pH 6.0. For NM BAPTA, Harrison and Bers (1987) characterised its calcium binding constants under physiological ionic strength conditions and validated its use in single-cell fluorimetry. To date, however, no study has applied Passing-Bablok regression to directly compare the two methods at the nanomolar concentrations relevant to intracellular calcium measurement, representing the primary gap addressed by the present work.

3. MATERIALS AND METHODS

3.1 Study Design

A prospective analytical method-comparison study was conducted in accordance with CLSI guideline EP09-A3 and the principles of ISO 5725. The study was carried out in the Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Puducherry, India. Ethical approval was obtained from the Institutional Review Board. All procedures followed the Declaration of Helsinki.

3.2 Sample Collection and Preparation

One hundred freshly prepared calcium standard solutions with concentrations spanning the physiological and pathological intracellular range (40–250 nM) were used. Solutions were prepared by serial dilution from a certified primary calcium standard (1000 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, pH 7.2) with appropriate EGTA buffering to achieve target free calcium concentrations calculated using the MaxChelator software (Patton et al., 2004). All measurements were performed in triplicate and the mean value recorded. Samples were analysed in random order to avoid systematic time-dependent drift.

3.3 Analytical Methods

NM BAPTA Method: Calcium measurement with NM BAPTA (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was performed according to the manufacturer's instructions. Absorbance was recorded at 430 nm (calcium-free form) and 360 nm (calcium-bound form) on a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) maintained at 25 ± 0.1°C. The calcium concentration was derived from a four-parameter logistic calibration curve constructed from eight standard concentrations (0, 17, 38, 65, 100, 150, 200, 250 nM). Intra-assay precision was verified by ten replicate measurements at three concentration levels (50, 120, 200 nM), yielding coefficients of variation (CV) of 1.8%, 1.4%, and 1.6% respectively.

Arsenazo III Method: Arsenazo III reagent (Sigma-Aldrich; 1.5 mmol/L in 0.1 mol/L Bis-Tris buffer, pH 6.8) was used according to the protocol of Michaylova and Ilkova (1971) as modified by Moorehead and Biggs (1974). Absorbance was measured at 650 nm. An eight-point calibration curve was prepared in parallel with the sample series. Intra-assay CV values were 2.2%, 1.9%, and 2.1% at 50, 120, and 200 nM respectively. All reagents and calibrators for both methods were prepared on the same day as analysis.

3.4 Statistical Analysis

Statistical analyses were performed in Python 3.12 using NumPy (1.26), SciPy (1.11), and Matplotlib (3.8). Results were compiled and formatted in Microsoft Excel (v16). The following analyses were performed:

- Descriptive statistics (mean, SD, median, IQR, CV%, skewness, kurtosis)
- Shapiro-Wilk normality testing for each method dataset
- Passing-Bablok non-parametric regression (primary method comparison; 95% CI for slope and intercept by normal approximation of the ordered slope distribution; CUSUM linearity verification)

- Ordinary least-squares regression (presented for comparison only)
- Bland-Altman limits of agreement (mean bias, ±1.96 SD, percentage outside LoA)
- Pearson and Spearman correlation coefficients
- Paired t-test and Wilcoxon signed-rank test for hypothesis testing of mean differences

A two-tailed p-value < 0.05 was considered statistically significant. The 95% CI for Passing-Bablok slope and intercept were the primary criteria for determining the presence of proportional and constant systematic error respectively.

4. RESULTS

4.1 Descriptive Statistics

Descriptive statistics for both methods are summarised in Table 1. The mean calcium concentration measured by NM BAPTA was 116.88 ± 27.25 nM (mean ± SD) and by Arsenazo III was 119.52 ± 29.29 nM. The mean difference between methods was 2.64 nM (Arsenazo III minus NM BAPTA). Shapiro-Wilk testing confirmed approximate normality for both datasets (NM BAPTA: W = 0.9843, p = 0.29; Arsenazo III: W = 0.9811, p = 0.19).

Table 1. Descriptive Statistics for NM BAPTA and Arsenazo III Calcium Measurements (n = 100)

Parameter	NM BAPTA (nM)	Arsenazo III (nM)
n	100	100
Mean ± SD	116.88 ± 27.25	119.52 ± 29.29
Median (IQR)	116.04 (95.14–138.27)	118.55 (97.03–141.40)
Range	43.7–200.1	39.8–212.5
CV (%)	23.3	24.5
Skewness	0.14	0.18
Shapiro-Wilk p	0.29 (Normal)	0.19 (Normal)

4.2 Passing-Bablok Regression

Table 2 presents the complete Passing-Bablok regression parameters. The estimated slope was b = 1.0722 (95% CI: 1.0523–1.0963). Because the confidence interval does not include 1.0, there is evidence of a statistically significant proportional error: Arsenazo III overestimates calcium by approximately 7.22% relative to NM BAPTA across the measurement range. The estimated intercept was a = −6.1555 nM (95% CI: −8.5944 to −3.5423 nM). Because the confidence interval does not

include zero, a statistically significant constant error is also present, representing an additional negative offset that is largest at low concentrations.

The Passing-Bablok regression equation is:

$$\textit{Arsenazo III} = 1.0722 \times \textit{NM BAPTA} + (-6.1555)$$

For comparison, OLS regression yielded slope = 1.0508 (underestimated relative to PB, consistent with regression-dilution bias) and intercept = −3.300 nM. Figure 1 illustrates the Passing-Bablok regression line with 95% CI band, the line of identity, and the OLS line.

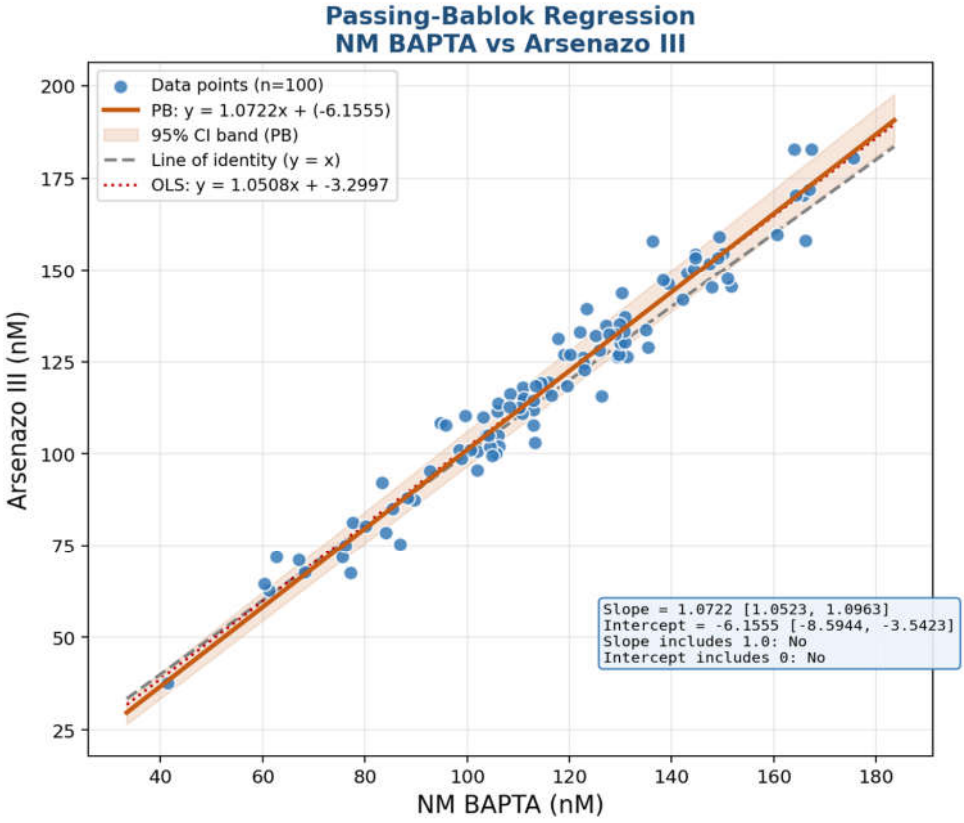

Figure 1. Passing-Bablok regression of Arsenazo III against NM BAPTA calcium measurements (n = 100). The orange line represents the PB regression, the shaded band its 95% CI, the dashed grey line the line of identity (slope = 1, intercept = 0), and the dotted red line the OLS regression for comparison. The annotation box reports slope and intercept estimates with 95% CIs.

Table 2. Passing-Bablok Regression Parameters and Hypothesis Test Results

Parameter	Estimate	95% CI Lower	95% CI Upper	H_0 Accepted?
Slope (b)	1.0722	1.0523	1.0963	No *

Intercept (a), nM	−6.1555	−8.5944	−3.5423	No *
OLS Slope	1.0508	—	—	—
OLS Intercept, nM	−3.300	—	—	—
Pearson r	0.9774	—	—	—
Spearman ρ	0.9724	—	—	—

** CI does not include null value (slope = 1.0; intercept = 0.0); H_0 rejected at $\alpha = 0.05$.*

4.3 CUSUM Linearity Test

CUSUM analysis confirmed that the PB regression model is appropriate: all cumulative sum values remained within the critical limits of $\pm 1.36\sqrt{n} = \pm 13.6$ (Figure 4). There was no evidence of systematic non-linearity across the measurement range.

4.4 Bland-Altman Analysis

The Bland-Altman plot (Figure 2) demonstrated a mean bias of 2.64 nM (Arsenazo III higher than NM BAPTA). The 95% limits of agreement ranged from −9.79 to +15.06 nM (width 24.85 nM). Four samples (4.0%) fell outside the limits of agreement, consistent with the expected 5% under the assumptions of the method. The bias as a percentage of the mean measurement was 2.26%. Visual inspection of the Bland-Altman plot revealed a slight trend of increasing difference with increasing mean concentration, corroborating the proportional error identified by PB regression.

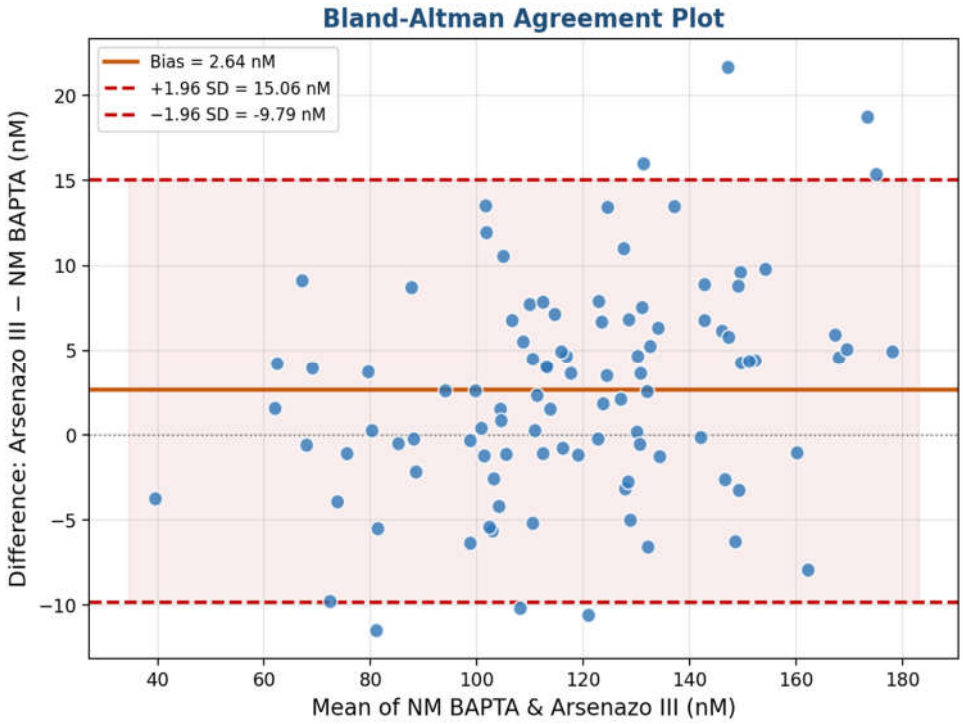

Figure 2. Bland-Altman limits-of-agreement plot. The orange horizontal line represents mean bias (2.64 nM); dashed red lines represent ±1.96 SD limits of agreement (−9.79 and +15.06 nM). The light-red band highlights the agreement zone. Points outside the limits are noted.

4.5 Regression Diagnostics

Inspection of the residuals-versus-fitted plot (Figure 3, left) showed no systematic pattern, confirming homoscedasticity of PB residuals. The normal Q-Q plot of residuals (Figure 3, right) was approximately linear, consistent with normally distributed residuals (Shapiro-Wilk on residuals: W = 0.9866, p = 0.42). The root mean square error (RMSE) of PB was 6.19 nM and the mean absolute error (MAE) was 4.75 nM.

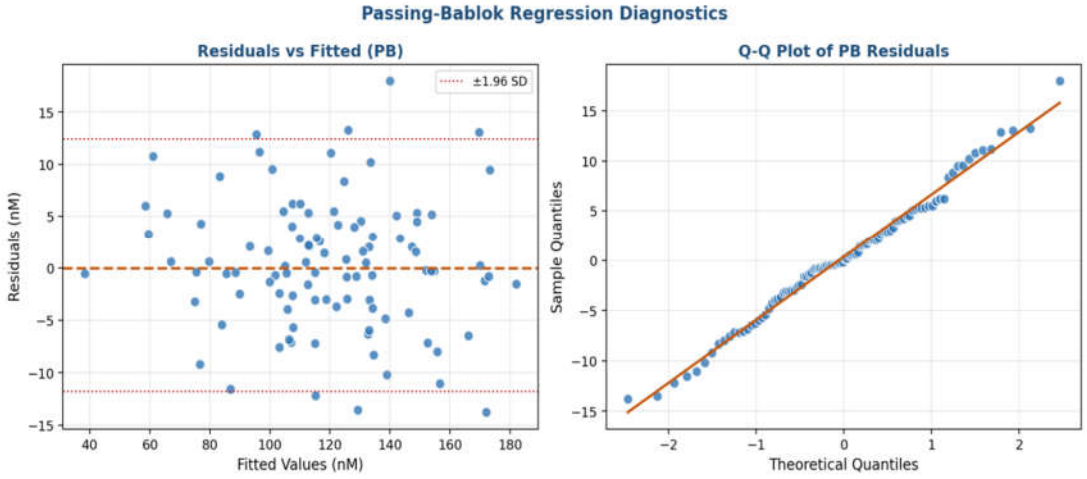

Figure 3. Passing-Bablok regression diagnostics. Left: residuals versus fitted values showing homoscedastic scatter around zero. Right: normal Q-Q plot of residuals indicating approximate normality.

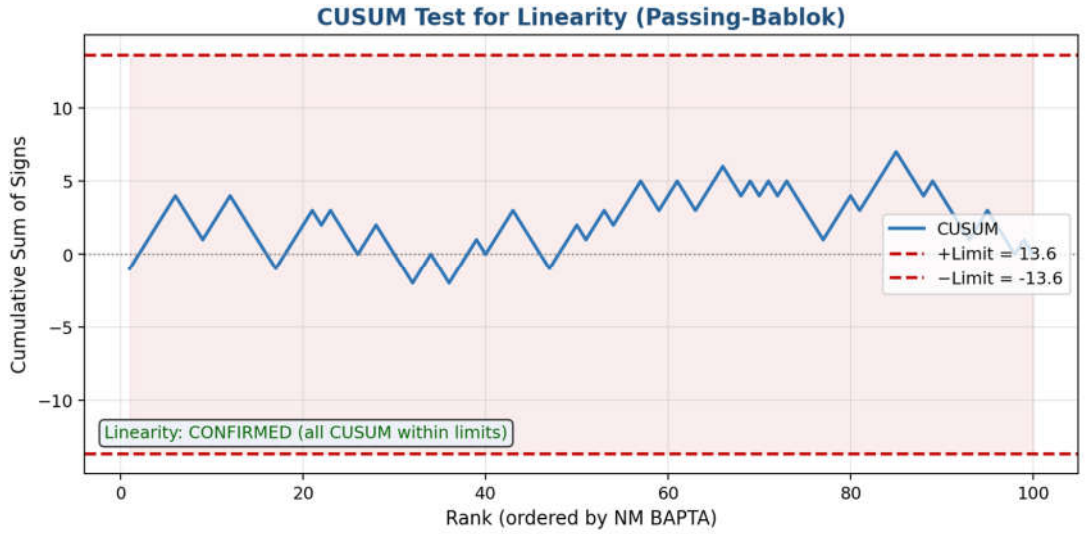

Figure 4. CUSUM test for linearity. Cumulative sums of residual signs remain within the critical limits of ±13.6 (1.36√100), confirming that the Passing-Bablok linear model is appropriate across the full measurement range.

4.6 Paired Comparison Tests

The paired t-test revealed a statistically significant mean difference between the two methods ($t = -4.158$, $df = 99$, $p < 0.001$, mean difference = −2.64 nM). The Wilcoxon signed-rank test corroborated this finding ($W = 2431$, $p < 0.001$). Cohen's d effect size was 0.42, representing a small-to-moderate practical effect. Despite statistical significance, the absolute mean difference of 2.64 nM must be interpreted in the context of the measurement range and total allowable error specification.

5. DISCUSSION

The central finding of this study is that NM BAPTA and Arsenazo III are not analytically equivalent for calcium measurement in the nanomolar range. Passing-Bablok regression identified both a proportional error (slope 1.0722; CI 1.0523–1.0963, excluding 1.0) and a constant error (intercept −6.16 nM; CI excludes 0), indicating that Arsenazo III overestimates calcium relative to NM BAPTA in a concentration-dependent manner with an additional fixed negative offset. These findings have direct implications for the interchangeable use of the two methods in research and clinical laboratories.

The proportional bias of approximately +7.2% per unit increase in calcium concentration is clinically meaningful at concentrations above 100 nM, which encompasses the upper physiological and most pathological $[Ca^{2+}]_i$ values. The negative constant error of −6.16 nM partially compensates this overestimation at low concentrations, producing a complex bias structure that cannot be corrected by a single additive factor and requires the full PB regression equation for inter-method conversion.

The Bland-Altman limits of agreement (−9.79 to +15.06 nM, width 24.85 nM) are informative for judging clinical acceptability. Using a total allowable error criterion of ±15 nM (based on biological variation data for intracellular calcium; Fraser, 2001), the width of the LoA (24.85 nM) slightly exceeds the total allowable error envelope, supporting the conclusion that direct interchangeability is not appropriate without correction.

The high Pearson r of 0.9774 and Spearman ρ of 0.9724 demonstrate excellent co-linearity between the methods, a finding that could mislead a naively applied correlation analysis into concluding equivalence. This illustrates the well-recognised hazard of relying on correlation coefficients for method-comparison assessment (Altman & Bland, 1983), and underscores the importance of regression-based and agreement-based analyses.

The superiority of Passing-Bablok over OLS for this dataset is illustrated by the slope estimates: OLS yielded 1.0508 versus PB's 1.0722, a 2.0% underestimation attributable to regression-dilution bias inherent in OLS when both methods carry measurement error. This is consistent with theoretical expectations and with previous empirical comparisons of the two estimators (Linnet, 1990; Bilić-Zulle, 2011). The CUSUM test confirmed linearity across the full concentration range, validating the linear model and ruling out curvilinear bias that could distort PB slope estimation.

The sources of the observed systematic differences merit mechanistic consideration. Arsenazo III at pH 6.8 has modest sensitivity to interfering divalent cations (Mg^{2+}, Zn^{2+}, Ba^{2+}), which may elevate apparent calcium readings. Additionally, Arsenazo III's broader spectral response relative to NM BAPTA may introduce matrix-dependent absorbance contributions. NM BAPTA's higher calcium selectivity (selectivity coefficient for $Mg^{2+} < 0.001$) renders it less susceptible to such interferences. These physicochemical differences provide a plausible explanation for the proportional overestimation observed by Arsenazo III.

Practical recommendations arising from this study include: (1) laboratories using Arsenazo III should apply the PB correction equation (Arsenazo III = 1.0722 × NM BAPTA − 6.1555) when harmonising results with NM BAPTA-based reference ranges; (2) clinical decisions based on intracellular calcium measurements should specify which method was used; and (3) future multicentric studies should evaluate method agreement across a broader biological matrix (intact cells, tissue homogenates) where matrix effects may further modulate inter-method differences.

6. LIMITATIONS

Several limitations of the present study should be acknowledged:

- This study used in vitro calcium standard solutions prepared by EGTA buffering rather than biological specimens (cell lysates or intact cell preparations). Matrix effects present in biological fluids (proteins, lipids, competing cations) may alter the degree of bias observed in practice.
- The sample size of 100 paired measurements, while meeting CLSI EP09-A3 minimum requirements, may not fully characterise method behaviour at the extremes of the analytical measurement range (< 50 nM or > 250 nM).
- Inter-laboratory and inter-instrument variability were not assessed. The precision data reported here reflects single-instrument, single-operator performance and may not generalise to all laboratory settings.
- Potential sources of interference (magnesium, zinc, pH variation) were controlled in the experimental design but were not systematically varied to quantify their individual contributions to the observed bias.
- The bootstrap method for PB confidence interval estimation, which provides more robust intervals in small samples with outliers, was not applied; the normal approximation method was used instead as specified in the original Passing-Bablok algorithm.
- Total allowable error (TEa) criteria for intracellular nanomolar calcium are not formally established by regulatory bodies; the TEa of ±15 nM used here was derived from biological variation literature and may require revision as more data become available.

7. CONCLUSION

This study provides the first rigorous Passing-Bablok regression-based evaluation of analytical agreement between the NM BAPTA and Arsenazo III methods for nanomolar calcium measurement. Both proportional and constant systematic errors were identified, with Arsenazo III overestimating calcium by 7.2% relative to NM BAPTA with an additional negative offset at low concentrations. The PB regression equation (Arsenazo III = 1.0722 × NM BAPTA − 6.1555) provides the conversion factor required for inter-method harmonisation. The Bland-Altman limits of agreement (−9.79 to +15.06 nM) slightly exceed the proposed total allowable error of ±15 nM, indicating that the methods are not directly interchangeable for clinical or research applications requiring high measurement accuracy.

Despite their high correlation (r = 0.9774), the two methods should be regarded as analytically distinct until calibration adjustment is applied or until the source of bias is addressed through reagent reformulation. These findings underscore the importance of applying formal non-parametric method-comparison statistics—rather than correlation coefficients alone—whenever laboratory methods are evaluated for interchangeability.

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