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ANALYTICAL METHOD VALIDATION REPORT: BIS-TRIS HCL ASSAY BY POTENTIOMETRIC TITRATION

TABLE OF CONTENTS

1. PURPOSE:

1.1. The purpose of this Report is to:

1.1.1. Provide performance data demonstrating that the Bis-Tris HCl Assay via Potentiometric Titration procedure is adequately evaluated and validated.

1.1.2. Provide validation that the Bis-Tris HCl Assay via Potentiometric Titration procedure meets all requirements for accuracy, precision, linearity, range, specificity, and intermediate precision.

2. SCOPE:

2.1. This Analytical Method Validation Report applies to the Bis-Tris HCl Assay by Potentiometric Titration with standardized 0.1N Sodium Hydroxide.

2.2. The Bis-Tris HCl Assay via Potentiometric Titration was validated as a Category I (Quantitative) method.

2.3. The scope of this method validation protocol covers Bis-Tris HCl content for a 0.75g sample size from a range of 25.0%-120.0%.

2.4. The approximate standardization volume of the titrant determined the 100% target sample size.

2.4.1. Standardization of the 0.1N NaOH was performed with 0.6g of traceable Potassium Hydrogen Phthalate (KHP), (204.22g/mol) material; equivalent to ~3mmol.

2.4.2. 100% Assay level is 0.75g of Bis-Tris HCl (245.70g/mol) Sample; equivalent to ~3mmol.

3. RESPONSIBILITIES:

3.1. The Associate Director of Product Lifecycle and/or qualified designee was responsible for completing the Method Validation Report using conclusions made for the results obtained during testing.

3.2. The Quality Control Manager and/or qualified designee is responsible for the control, training, implementation, and maintenance of this report.

3.3. The Laboratory Analyst(s) and/or qualified designee(s) were responsible for performing the testing stated in the validation protocol.

4. REFERENCE:

4.1. BSI-PRL-0579, Analytical Method Validation Protocol: Bis-Tris HCl Assay by Potentiometric Titration

4.2. BSI-SOP-0098, Balance SOP.

4.3. BSI-SOP-0126, Laboratory Notebooks.

4.4. BSI-SOP-0140, Standardization of Titrants

4.5. BSI-SOP-0143, Metrohm Titrando 907 Auto-Titrator SOP.

4.6. BSI-SOP-0436, Analytical Methods Validation Master Plan.

5. PRE-VALIDATION REQUIREMENTS:

5.1. Equipment

5.1.1. All equipment used in this Validation was in proper working order and with current calibrations. Serial numbers, date of last calibration, and the calibration due date for each instrument / equipment, where applicable, are included in this report.

5.2. Personnel

5.2.1. All personnel who executed this validation were properly trained on the analysis technique in accordance with the Analytical Methods Validation Master Plan.

5.3. Supplies

5.3.1. Any supplies to be used in the Validation were clean and appropriate for the intended use. All supplies are listed in the Materials and Equipment section of this report.

5.4. Reagents
5.4.1. All reagents were current, met required specifications and were suitable for the intended use. The reagent name, lot number, manufacturer, date of opening, date of expiration, and part number are included in this report.
5.5. Reference Standards
5.5.1. Any standards used in this Validation are listed in the Materials and Equipment section of this report. The name of the reference standard, lot number, manufacturer, date of opening, date of expiration, and part number are included in this report.

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this validation are outlined in this section.
6.2. Equipment
6.2.1. Analytical Balance
6.2.2. Titrando 907 Auto-titrator equipped with pH electrode and 50mL burette

Equipment and Instrumentation					
Equipment	**Model / Part Number**	**Manufacturer**	**Serial Number**	**Calibration Due Date**	**Date of Last Calibration**
Auto-Titrator	907 Titrando	Metrohm	12155	7/24	7/28/23
Analytical Balance	Secura124-1S	Sartorius	29212172	10/31/23	4/20/23
pH Probe	6.0278.300	Metrohm	20283243	Not Applicable	Not Applicable

6.3. Reagents and Standards
6.3.1. 0.1N NaOH
6.3.2. Purified Water
6.3.3. Potassium Hydrogen Phthalate (KHP) Standard

Reagents and Standards						
Reagent / Standard	**Lot ID**	**Manufacturer**	**Part Number**	**CAS**	**Expiry Date**	**Date of Opening**
Bis-Tris HCl	SLCJ3337	Sigma Aldrich	B6032-500G	124763-51-5	4/7/27	7/29/22
Traceable KHP Reference Standard	BSP38P54	In-House	Not Applicable	877-24-7	10/26/23	7/27/23
Purified Water	F9SA14284H	Millipore Sigma	Not Applicable	7732-18-5	12/31/23	6/7/23
0.1N Sodium Hydroxide	226652	Fisher Chemical	SS276-4	1310-73-2 7732-18-5	2/25	8/7/23

6.4. Supplies
6.4.1. Graduated Cylinders
6.4.2. Assorted Beakers
6.4.3. Weight paper/funnel
6.4.4. Stir bar

7. PROCEDURE:

7.1. Standardize 0.1N NaOH as per Standardization of Titrants (DCN: BSI-SOP-0140).

7.1.1. Note: Calibrate pH probe per Standardization of Titrants (DCN: BSI-SOP-0140) if pKa is to be reported.

7.2. Bis-Tris HCl Sample: If in the form of large crystals, grind 10g of Bis-Tris HCl to homogenize.

7.3. Sample Analysis and Validation Testing Matrix:

7.3.1. Accurately weigh (see Sample Preparations Table) grams of sample into a suitable beaker.

7.3.2. Add 50mL of water.

7.3.3. Titrate to a potentiometric endpoint.

7.3.4. Calculate % Bis-Tris HCl using the following equation in the Metrohm® Tiamo™ software:

$$\%Bis-Tris\ HCl = \frac{(mL\ NaOH)(N\ NaOH)(24.570)}{Sample\ Weight\ (g)}$$

7.3.5. Record Result.

7.4. Sample Preparations

SAMPLE PREPARATIONS

ANALYST I SAMPLE PREPARATION TABLE

Sample ID/Purpose		Bis-Tris HCl Weight (g)	Approximate 0.1N NaOH Volume Consumed	% Burette Volume Used	Analysis Level (%)
Blank/ Specificity	1	0.000			0%
	2	0.000			
	3	0.000			
Linearity 1	1	0.19	8mL	16%	25%
Accuracy Precision Linearity 2	1	0.38	15mL	30%	50%
	2	0.38			
	3	0.38			
Accuracy Precision Linearity 3	1	0.60	24mL	48%	80%
	2	0.60			
	3	0.60			
Accuracy Precision Linearity 4	1	0.75	30mL	60%	100%
	2	0.75			
	3	0.75			
	4	0.75			
	5	0.75			
	6	0.75			
Accuracy Precision Linearity 5	1	0.90	36mL	72%	120%
	2	0.90			
	3	0.90			

ANALYST II SAMPLE PREPARATION TABLE

Sample ID/Purpose		Bis-Tris HCl Weight (g)	Approximate 0.1N NaOH Volume Consumed	% Burette Volume Used	Analysis Level (%)
Int. Precision	1	0.75	30mL	60%	100%
	2	0.75			
	3	0.75			
	4	0.75			
	5	0.75			
	6	0.75			

8. PERFORMANCE PARAMETERS:

8.1. Linearity
- 8.1.1. Linearity will be assessed across five (5) analysis levels listed in section 7.4.
- 8.1.2. Plot and report the Coefficient of Determination (r^2), slope, y-intercept, and residual sum of squares of the average endpoint volume (mL) to average weight of Bis-Tris HCl analyzed (g).
- 8.1.3. The Coefficient of Determination (r^2) of the linear regression line must be NLT 0.99.

8.2. Range
- 8.2.1. The range will be established by showing an acceptable degree of Linearity, Accuracy, and Precision.
- 8.2.2. A minimum range of 80% to 120% of the 100% test weight is required.

8.3. Accuracy and Precision:
- 8.3.1. Accuracy and Precision will be assessed using fifteen (15) determinations over four (4) Analysis Levels with six (6) determinations at the 100% Analysis Level.
- 8.3.2. Accuracy is assessed as Percent Recovery. All samples must have a percent recovery of 99.0% to 101.0%.
- 8.3.3. Precision is assessed as standard deviation (s), Relative Standard Deviation (%RSD), and the 95% Confidence Interval reported for each analysis level. Each Analysis Level must have a Relative Standard Deviation (%RSD) of NMT 1.0%.

8.4. Intermediate Precision
- 8.4.1. Intermediate Precision will be assessed by having a second analyst (Analyst II) perform a separate 0.1N NaOH standardization from Analyst I, prepare, and analyze six (6) determinations at the 100% Analysis Level on a separate day from Analyst I.
- 8.4.2. Intermediate Precision is assessed as Standard Deviation (s), Relative Standard Deviation (%RSD), and the 95% Confidence Interval reported. The Relative Standard Deviation (%RSD) of the individual and combined 100% Analysis Level results must be NMT 1.0%.

8.5. Specificity:
- 8.5.1. Sodium Hydroxide titrations are specific to acids.
- 8.5.2. Specificity will be demonstrated by acceptable accuracy and precision data and lack of meaningful response to triplicate analysis of the blank.

9. CALCULATIONS:

9.1. Percent Recovery

$$Percent\ Recovery\ (\%) = \frac{Calculated\ Assay\ Result\ (\%)}{Bis - Tris\ HCl\ CoA\ Assay\ Value\ (\%)} \times 100$$

9.2. Standard Deviation (s)

$$Standard\ Deviation\ (s) = \sqrt{\frac{\sum(X_i - \bar{X})^2}{n-1}}$$

Where:

X_i = Each Individual Bis-Tris HCl Assay Value (%)

$\bar{X}$ = Average Bis-Tris HCl Assay Value (%)

n = Number of Bis-Tris HCl Assay determinations

9.3. Relative Standard Deviation (%RSD)

$$Relative\ Standard\ Deviation\ (\%RSD) = \frac{Standard\ Deviation\ (\%)}{Average\ Bis - Tris\ HCl\ Assay\ Value\ (\%)} \times 100$$

9.4. 95% Confidence Interval

$$95\%\ Confidence\ Interval = \bar{X} \pm z\left(\frac{Standard\ Deviation\ (\%)}{\sqrt{n}}\right)$$

Where:

$\bar{X}$ = Average Bis-Tris HCl Assay Value (%)

z = 1.960 = Z Value at the 95% Confidence Interval

n = Number of Bis-Tris HCl Assay determinations

9.5. Residual Sum of Squares (RSS)

$$RSS = \sum(Actual\ Titrant\ volume - Theoretical\ Titrant\ Volume)^2$$

9.5.1. Theoretical Titer Volume is calculated using the average sample weight (g) from Step 11.5 and the Bis-Tris HCl CoA Assay Value (%) to back-calculate using the equation from Step 7.3.4.

10. VALIDATION SUMMARY:

Validation Summary		
Performance Parameter	**Acceptance Criteria**	**Results**
Standardization	• Titer Value: Report • Each replicate must be within ±0.0005 units of each other.	Analyst I: • Titer Value = 0.1002N • Replicate Deviation = 0.0001 Analyst II: • Titer Value = 0.1007N • Replicate Deviation = 0.0004
Accuracy	• All samples must have a percent recovery of 99.0% to 101.0%.	50% Level • Replicate 1 = 100.5% • Replicate 2 = 100.4% • Replicate 3 = 100.4% 80% Level • Replicate 1 = 100.1% • Replicate 2 = 100.2% • Replicate 3 = 100.3% 100% Level • Replicate 1 = 99.9% • Replicate 2 = 100.0% • Replicate 3 = 100.0% • Replicate 4 = 99.9% • Replicate 5 = 100.0% • Replicate 6 = 99.9% 120% Level • Replicate 1 = 99.9% • Replicate 2 = 99.9% • Replicate 3 = 100.1%

Validation Summary		
Performance Parameter	**Acceptance Criteria**	**Results**
Precision	• Each analysis level must have a %RSD of NMT 1.0%. • Standard Deviation: Report • 95% Confidence Interval: Report	50% Level • Standard Deviation = 0.098% • %RSD = 0.1% • 95% Confidence Interval = 0.111% 80% Level • Standard Deviation = 0.078% • %RSD = 0.1% • 95% Confidence Interval = 0.088% 100% Level • Standard Deviation = 0.050% • %RSD = 0.1% • 95% Confidence Interval = 0.040% 120% Level • Standard Deviation = 0.102% • %RSD = 0.1% • 95% Confidence Interval = 0.116%
Linearity	• Report the Coefficient of Determination (r^2), Slope, Y-Intercept, and Residual Sum of Squares. • The Coefficient of Determination (r^2) must be NLT 0.99.	• Coefficient of Determination (r^2) = 1.000 • Slope = 40.48 • Y-Intercept = 0.106 • Residual Sum of Squares = 0.010
Specificity	• Requirements for accuracy and precision are met. • There is lack of meaningful response to the triplicate analysis of the blank.	• Requirements for accuracy and precision were met. • There was no meaningful response in the triplicate analysis of the blank.
Range	• The range will be established by showing an acceptable degree of Linearity, Accuracy, and Precision at each level. • A minimum range of 80% to 120% of the 100% test weight is required.	• Range: 50% to 120%

Validation Summary		
Performance Parameter	**Acceptance Criteria**	**Results**
Intermediate Precision	• Report the individual and combined (Analyst I and II) Standard Deviation, %RSD, and 95% Confidence Interval. • The individual and combined (Analyst I and II) %RSD is NMT 1.0%.	Individual (Analyst II) • Standard Deviation = 0.176% • %RSD = 0.2% • 95% Confidence Interval = 0.141% Combined (Analyst I and II) • Standard Deviation = 0.164% • %RSD = 0.2% • 95% Confidence Interval = 0.032%

11. VALIDATION RESULTS:

11.1. **Standardization:**

11.1.1. Standardized 0.1N Sodium Hydroxide as per Standardization of Titrants (BSI-SOP-0140). Performed the standardization in triplicate using the average of the replicates to determine the normality of the titrant.

11.1.2. Acceptance Criteria:

11.1.2.1. Each replicate of the standardization must be within ±0.0005 units of the others to be considered acceptable.

Analyst 1: 0.1N NaOH Standardization			
Replicate	**KHP Standard Weight (g)**	**Titrant Volume (mL)**	**Result (N)**
1	0.6035	29.4929	0.1002
2	0.6755	33.0162	0.1002
3	0.6136	30.0083	0.1001
		Average	0.1002
		Deviation	0.0001

Analyst II: 0.1N NaOH Standardization			
Replicate	**KHP Standard Weight (g)**	**Titrant Volume (mL)**	**Result (N)**
1	0.6014	29.1791	0.1009
2	0.6037	29.3652	0.1007
3	0.6031	29.3978	0.1005
		Average	0.1007
		Deviation	0.0004

11.2. **Accuracy:**

11.2.1. Accuracy was assessed using fifteen (15) determinations over four (4) analysis levels with six (6) determinations at the 100% Analysis Level.

11.2.2. Accuracy was assessed by comparing the Bis-Tris HCl Assay Values to the Bis-Tris HCl Certified Assay Value found on the Certificate of Analysis and calculating the percent recovery.

11.2.3. Acceptance Criteria:

11.2.3.1. All samples must have a percent recovery of 99.0% to 101.0%.

Accuracy Results			
		Bis-Tris HCl Certified Assay Value:	100.0%
Analysis Level (%)	**Replicate**	**Bis-Tris HCl Assay Result (%)**	**Percent Recovery (%)**
50%	1	100.54	100.5
	2	100.37	100.4
	3	100.37	100.4
80%	1	100.13	100.1
	2	100.24	100.2
	3	100.28	100.3
100%	1	99.92	99.9
	2	100.00	100.0
	3	99.97	100.0
	4	99.86	99.9
	5	99.97	100.0
	6	99.93	99.9
120%	1	99.86	99.9
	2	99.89	99.9
	3	100.05	100.1

11.3. **Precision:**

11.3.1. Precision was assessed using fifteen (15) determinations over four (4) analysis levels with six (6) determinations at the 100% Analysis Level.

11.3.2. Precision was assessed by calculating the Standard Deviation, Relative Standard Deviation (%RSD), and 95% Confidence Interval at each analysis level.

11.3.3. Acceptance Criteria:

11.3.3.1. Standard Deviation: Report.

11.3.3.2. Relative Standard Deviation (%RSD): NMT 1.0%.

11.3.3.3. 95% Confidence Interval: Report.

Precision Results					
Analysis Level (%)	**Replicate**	**Bis-Tris HCl Assay Result (%)**	**Standard Deviation (%)**	**Relative Standard Deviation (%RSD) (%)**	**95% Confidence Interval (%)**
50%	1	100.54	0.098	0.1	0.111
	2	100.37			
	3	100.37			
80%	1	100.13	0.078	0.1	0.088
	2	100.24			
	3	100.28			
100%	1	99.92	0.050	0.1	0.040
	2	100.00			
	3	99.97			
	4	99.86			
	5	99.97			
	6	99.93			
120%	1	99.86	0.102	0.1	0.116
	2	99.89			
	3	100.05			

11.4. **Linearity:**

11.4.1. The average endpoint volumes at each concentration from the Accuracy / Precision portion of this report were analyzed to determine linearity over a range of five (5) analysis levels.

11.4.2. Plotted the average endpoint volume (mL) to the average Bis-Tris HCl sample weight (g) and reported the Coefficient of Determination (r^2), Slope, Y-Intercept, and Residual Sum of Squares.

11.4.3. Acceptance Criteria:

11.4.3.1. Coefficient of Determination (r^2): NLT 0.99

11.4.3.2. Slope: Report

11.4.3.3. Y-Intercept: Report

11.4.3.4. Residual Sum of Squares: Report

Linearity Results						
Analysis Level (%)	**Average Bis-Tris HCl Weight (g)**	**Average Endpoint Volume (mL)**	**Coefficient of Determination (r^2)**	**Slope**	**Y-Intercept**	**Residual Sum of Squares**
25	0.1926	7.8841	1.0000	40.48	0.106	0.010
50	0.3814	15.5582				
80	0.6011	24.4698				
100	0.7530	30.5697				
120	0.9030	36.6522				

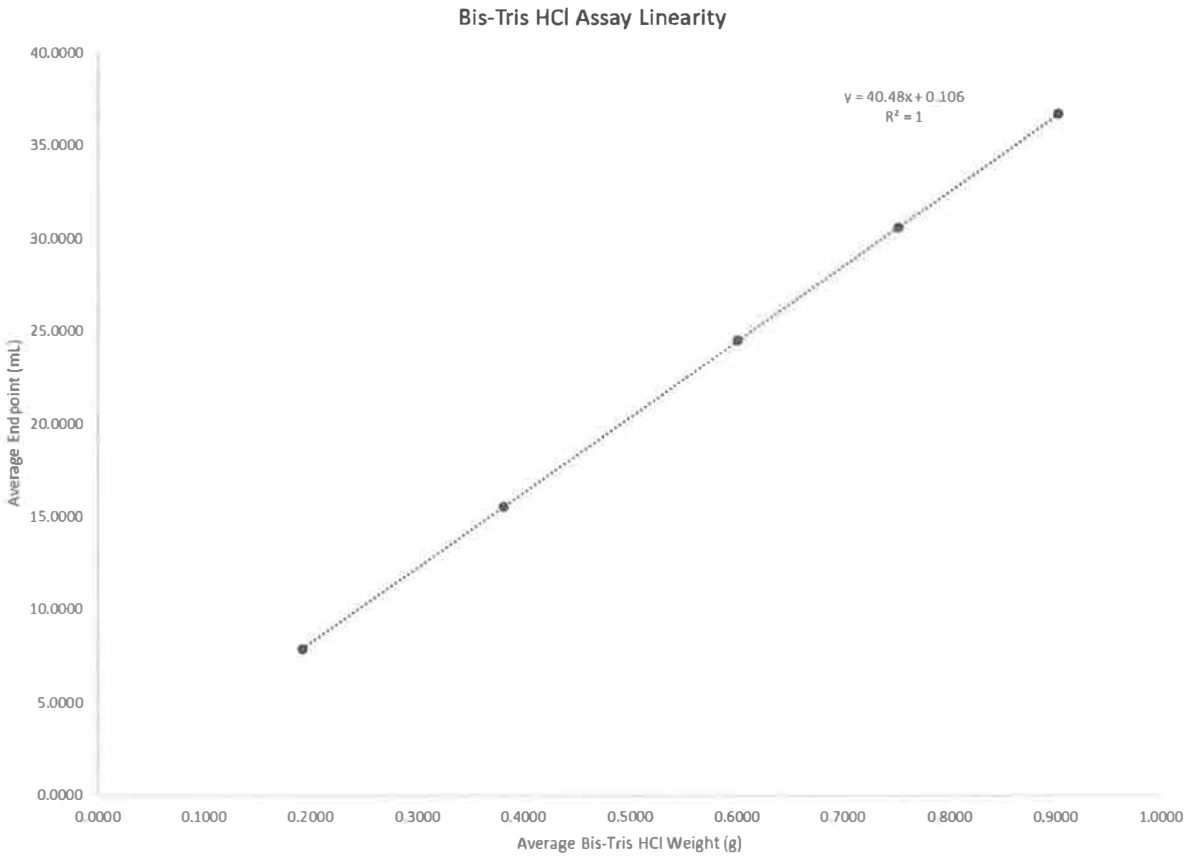
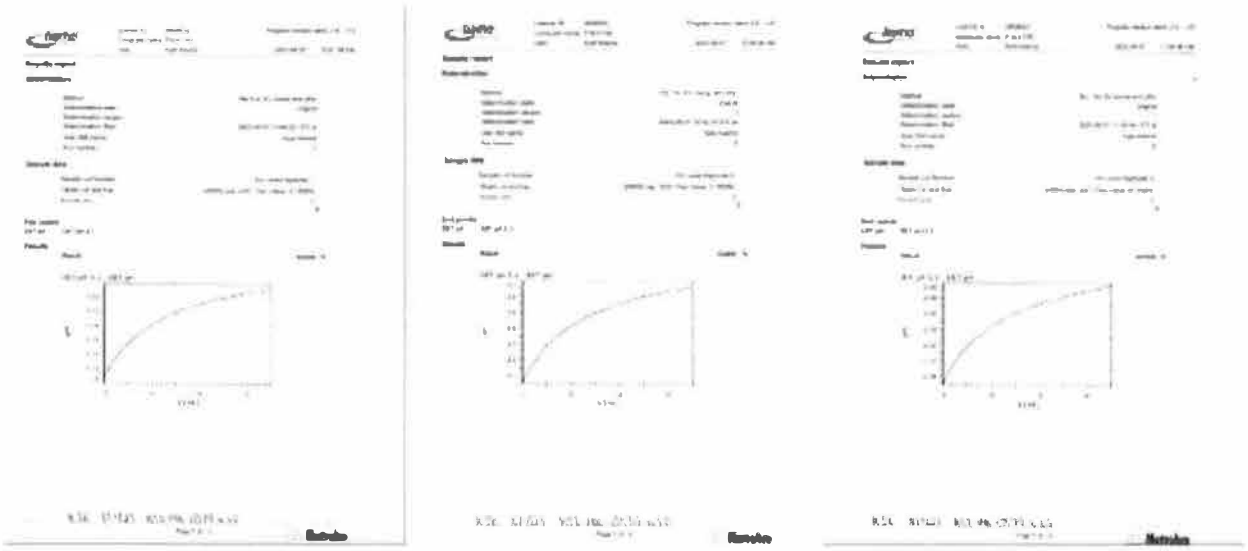


Figure 1: Bis-Tris HCl Assay Linearity – Average Bis-Tris HCl Weight (grams) vs. Average Endpoint Volume (mL)

11.5. **Specificity:**

11.5.1. Specificity was demonstrated by meeting requirements for Accuracy and Precision and lack of meaningful response to triplicate analysis of the blank.



Specificity Results	
Acceptance Criteria	**Result**
Requirements for Accuracy were met.	Pass
Requirements for Precision were met.	Pass
There was lack of meaningful response to the triplicate analysis of the blank.	Pass

11.6. **Range:**

11.6.1. The range of the method is defined as the specified range that demonstrates an acceptable degree of linearity, accuracy, and precision at each level.

11.6.2. Acceptance Criteria:

11.6.2.1. A range of 80% to 120% of the 100% test weight is required.

Range of Analysis: 50% to 120%

11.7. **Intermediate Precision:**

11.7.1. Intermediate Precision was assessed by having a second analyst (Analyst II) perform a separate 0.1N NaOH standardization from Analyst I, prepare, and analyze six (6) determinations at the 100% Analysis Level on a separate day from Analyst I.

11.7.2. Intermediate Precision was assessed by calculating Standard Deviation (s), Relative Standard Deviation (%RSD), and the 95% Confidence Interval.

11.7.3. Acceptance Criteria:

11.7.3.1. Standard Deviation (Individual and Combined): Report.

11.7.3.2. Relative Standard Deviation (Individual and Combined): NMT 1.0%.

11.7.3.3. 95% Confidence Interval (Individual and Combined): Report.

Intermediate Precision Results						
Analysis Level (%)	**Analyst**	**Replicate**	**Bis-Tris HCl Assay (%)**	**Standard Deviation (%)**	**Relative Standard Deviation (%RSD) (%)**	**95% Confidence Interval (%)**
100%	Analyst I	1	99.92	0.050	0.05	0.040
		2	100.00			
		3	99.97			
		4	99.86			
		5	99.97			
		6	99.93			
	Analyst II	1	100.41	0.18	0.2	0.141
		2	100.06			
		3	99.97			
		4	100.11			
		5	100.02			
		6	100.32			
			Combined:	0.164	0.2	0.032

12. CONCLUSION:

12.1. Performance Summary

Performance Summary	
Method Performance Indicator	**Result**
Standardization	Pass
Accuracy	Pass
Precision	Pass
Linearity	Pass
Specificity	Pass
Range	50% to 120% of 100% Sample Weight
Intermediate Precision	Pass

12.2. **Statement of Validation:** The method of analysis of Bis-Tris HCl Assay by Potentiometric Titration is considered a validated method of analysis at the Bangor, PA BioSpectra Inc. facility and is approved for use.