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ANALYTICAL METHOD VALIDATION REPORT: TROMETHAMINE ASSAY AND UNSPECIFIED DEGRADATION PRODUCTS VIA GC-FID

TABLE OF CONTENTS

1. PURPOSE:

1.1. The purpose of this report is to:

1.1.1. Ensure that the Tromethamine Assay and Unspecified Degradation Products determination via GC-FID procedure is adequately evaluated and validated.

1.1.2. Provide proof that the Tromethamine Assay and unspecified degradation products determination via GC-FID procedure meets all requirements for:

1.1.2.1. System Suitability
1.1.2.2. Accuracy
1.1.2.3. Precision
1.1.2.4. Linearity
1.1.2.5. Specificity
1.1.2.6. Range
1.1.2.7. Limit of Quantification (LOQ)/ Limit of Detection (LOD)
1.1.2.8. Solution Stability

2. SCOPE:

2.1. This Analytical Method Validation Report applies to the Tromethamine Assay and Unspecified Degradation Products determination via GC-FID method validation protocol.

2.2. This assay and degradation method will be considered as a Category I and II quantitative test respectively.

2.2.1. The Analytical Method Validation Master Plan dictates that this report will include assessment and conclusive statements of validation on the following: System Suitability, Accuracy, Precision, Specificity, Linearity, LOQ/LOD, Range, and Solution Stability.

2.2.2. System Suitability is required to be run for each analysis. Data was not reportable if system suitability does not meet requirements. An example system suitability is demonstrated in section 8; however, during this validation execution 5 system suitability and 2 robustness system suitability sets were analyzed. All met requirements and are detailed in the associated laboratory notebook pages.

3. RESPONSIBILITIES:

3.1. The Director of Laboratory Systems is responsible for the control, implementation, and maintenance of this report.

3.2. Analytical chemists who executed the validation protocol, with help and training from the Director of Laboratory Services and/or the Laboratory Manager, if necessary, were responsible for completing the Method Validation Report using conclusions made from the results obtained from testing.

4. REFERENCES:

4.1. BSI-ATM-0111, Assay of Tromethamine Via GC-FID
4.2. BSI-ATM-0112, Tromethamine Unspecified Degradation Products Via GC-FID
4.3. BSI-PRL-0688, Analytical Method Validation Protocol: Tromethamine Assay and Degradation Products via GC-FID
4.4. BSI-SOP-0098, Balance SOP
4.5. BSI-SOP-0126, Laboratory Notebooks
4.6. BSI-SOP-0134, Pipette SOP
4.7. BSI-SOP-0244, VWR Gravity Convection Operation and Calibration (Model Number: 414005-106)
4.8. BSI-SOP-0316, Shimadzu QP2010S GC/MS SOP
4.9. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.10. ICH Guideline for Analytical Validation Q2 (R1) and Q2 (R2)
4.11. ICH Guidelines for Impurities in New Drug Substances Q3A
4.12. USP NF <621>

5. EQUIPMENT:

5.1. Equipment
  5.1.1. All equipment used in this validation was in proper working order and within calibration.
5.2. Personnel
  5.2.1. All personnel were properly trained in accordance with the Analytical Methods Validation Master Plan.
5.3. Supplies
  5.3.1. All supplies used in the validation were clean and appropriate for their intended use.
5.4. Reagents
  5.4.1. All reagents were current and suitable for their intended use.
5.5. Reference Standards
  5.5.1. All standards that were used in this validation protocol were current and are listed in Section 6 below.
5.6. Method
  5.6.1. GC-2010
    5.6.1.1. Column Oven Temperature: 150.0°C
    5.6.1.2. Injection Mode: Split
    5.6.1.3. Injector temperature: 220.0°C
    5.6.1.4. Detector temperature: 275.0°C
    5.6.1.5. Flow Control Mode: Linear Velocity
    5.6.1.6. Pressure: 25.0 kPa
    5.6.1.7. Total Flow: 23.3 mL/min (Impurity Level) and 236.8 mL/min (Assay Level)
    5.6.1.8. Column Flow: 3.05 mL/min
    5.6.1.9. Linear Velocity: 29.2 cm/sec
    5.6.1.10. Purge Flow: 5.0 mL/min
    5.6.1.11. Split Ratio: 5 (Impurity level) and 75 (Assay level)

5.6.1.12. Note: The split (75) for the assay level is optimized for principle peak shape, while the reduced split (5) for the impurity level analysis is optimized for sensitivity to meet the detection requirements of the analysis.
5.6.1.13. High Pressure Injection: OFF
5.6.1.14. Carrier Gas Saver: OFF
5.6.1.15. Splitter Hold: OFF
5.6.1.16. Oven Temp Program:

Table 1: Oven Temperature Program

Rate (^{O}C per Min)	Temperature (°C)	Hold Time (min)
-	150.0	3.00
10.00	190.0	1.00
30.00	270.0	2.00
0.00	0.00	0.00

5.6.2. Ready Checks
5.6.2.1. Column Oven: YES
5.6.2.2. HS: NO
5.6.2.3. FID: YES
5.6.2.4. HS Carrier: NO
5.6.2.5. HS Purge: NO
5.6.2.6. APC1: YES
5.6.2.7. FID Makeup: YES
5.6.2.8. FID1 H2: YES
5.6.2.9. FID1 Air: YES
5.6.2.10. External Wait: NO
5.6.2.11. Auto Flame On: YES
5.6.2.12. Auto flame Off: YES
5.6.2.13. Reignite: YES
5.6.2.14. Auto Zero After Ready: YES
5.6.2.15. Equilibrium Time: 0.0 min

6. MATERIALS AND EQUIPMENT:

6.1. Instrumentation and Equipment
6.1.1. Analytical Balances

Table 2: Analytical Balances

Manufacturer	Model	Serial Number	Next Due	Last Service
Sartorius	MSE224S	24801744	10/31/23	04/20/23
Sartorius	Secura 124-1S	29212172	10/31/23	04/20/23
A&D	BM-20	T1004421	10/31/23	04/20/23

6.1.2. Micropipettes

Table 3: Micropipettes

Manufacturer	Model	Serial Number	Next Due	Last Service
Eppendorf	Research Plus	O39512B	06/30/23	12/22/22
Eppendorf	Research Plus	K53394I	06/30/23	12/22/22
Eppendorf	Research Plus	I45595H	11/30/23	05/23/23
Eppendorf	Research Plus	Q28940G	07/31/23	01/17/23
Eppendorf	Research Plus	L21310F	07/31/23	01/17/23
Eppendorf	Research Plus	J18397D	08/31/23	02/22/23
Eppendorf	Research Plus	G26211D	11/30/23	05/23/23
Eppendorf	Research Plus	R14419C	08/31/23	02/23/23
Eppendorf	Research Plus	R24330H	07/31/23	01/17/23
Eppendorf	Research Plus	Q31264C	08/31/23	02/22/23

6.1.3. Equipment

Table 4: Equipment

Manufacturer	Model	Serial Number	Next Due	Last Service
Shimadzu	GC-2010	020385050364	09/2023	09/13/22
VWR	Convection Oven	1100001176D009	08/31/23	05/23/23

6.1.4. Analytical GC Column

6.1.4.1. 30 m RTX-5 Amino column 0.53mm ID 1.00µm film thickness

6.1.4.1.1. Manufacturer: Restek; Model: 12355; Serial Number: 1646341

6.2. Reagents

Table 5: Reagents

Name	Supplier	Part No.	Lot	Due Date/Expiry	Open Date
Water	Milli-Q	Type 1 Ultra-Pure	F9SA14284H	12/31/23	Not Applicable
Bromocresol Purple TS	Not Applicable – In-House Solution	Not Applicable – In-House Solution	BSP36P55	02/2024	02/27/23
30% H_2O_2	Fisher Chemical	H325-500	226589	02/28/25	04/28/23
0.1N HCl	Fisher Chemical	SA54-4	231566	04/25	05/23/23
0.1N NaOH	Fisher Chemical	SS276-4	226735	01/25	05/12/23
Methanol	JT Baker	9093-03	22A2862003	01/18/27	06/29/23; 08/23/22

6.3. Reference Standard

Table 6: Reference Standard

Name	Supplier	Part No.	Lot	Expiry	Open Date
Tris	NIST	723e	723e	12/28/23	08/31/21

6.3.1. Supplies

6.3.1.1. Micropipette Tips: Eppendorf

6.3.1.2. Polypropylene weigh boats: TWD Scientific, LLC

6.3.1.3. Transfer Pipettes: Fisherbrand

7. PROCEDURE:

7.1. Solution Preparation – System Suitability Solutions

7.1.1. Diluent (6% Water in Methanol)

7.1.1.1. Pipetted 3 mL of water into a 50 mL volumetric flask and diluted to volume with methanol. Mixed thoroughly.

7.1.2. Assay Standard Solution (20 mg/mL Tromethamine)

7.1.2.1. Accurately weighed 1.00 g of Tromethamine CRS and transferred into a 50 mL volumetric flask. Pipetted in 3 mL of water, mixed, diluted to volume with methanol and mixed well.

7.1.2.2. Prepared in duplicate.

7.1.2.3. Labeled SS1 and SS2, respectively.

7.1.2.4. Retained SS2 for Solution Stability.

7.1.3. Unspecified Impurity-level Standard Solution (0.2 mg/mL Tromethamine)

7.1.3.1. Pipetted 5 mL of the SS1 solution into a 50 mL volumetric flask, added 3 mL of water, diluted to volume with methanol and mixed well.

7.1.3.2. Pipetted 5 mL of the solution prepared in Step 7.1.3.1. into a 50 mL volumetric flask, added 3 mL of water, diluted to volume with methanol, and mixed well.

7.1.3.3. Labeled flask Unspecified Impurity-level Standard Solution

7.1.4. LOQ Solution (0.02 mg/mL Tromethamine)

7.1.4.1. Pipetted 5 mL of the Unspecified Impurity-level Standard into a 50 mL volumetric flask, added 3 mL of water, diluted to volume with methanol, and mixed well.

7.1.4.2. Labeled flask: LOQ Solution

7.2. Solution Preparation – Stress Study

7.2.1. Acid Hydrolysis (20 mg/mL Tromethamine)

7.2.1.1. Transferred 200 mg of Tromethamine into a 10 mL volumetric flask, pipetted 0.3 mL of 0.1N Hydrochloric Acid into the flask, and stoppered the flask.

7.2.1.2. Placed the solution in an oven set at 40°C for 5 days.

7.2.1.3. After 5 days, pipetted 0.3 mL of 0.1N Sodium Hydroxide into the flask, diluted to volume with methanol and mixed.

7.2.2. Basic Hydrolysis (20 mg/mL Tromethamine)

7.2.2.1. Transferred 200 mg of Tromethamine into a 10 mL volumetric flask, pipetted 0.3 mL of 0.1N Sodium Hydroxide into the flask, and stoppered the flask.

7.2.2.2. Placed the solution in an oven set at 40°C for 5 days.

7.2.2.3. After 5 days, pipetted 0.3 mL of 0.1N Hydrochloric acid into the flask, diluted to volume with methanol and mixed.

7.2.3. Photolytic Sample (20 mg/mL Tromethamine)

7.2.3.1. Prepared in duplicate.

7.2.3.2. Transferred 200 mg of Tromethamine into a crystal dish, pipetted 0.6 mL of water to the dish and dissolved.

7.2.3.3. Exposed one (1) of the solutions to 1.2 million lux hours.

7.2.3.4. Kept the second solution (Control) in the dark until solution 1 had reached 1.2 million lux hours.

7.2.3.5. Carefully added 9.4 mL of methanol to the dish and mixed. Transferred the solution into a 10 mL volumetric flask.

7.2.4. Thermal Sample (20 mg/mL Tromethamine)

7.2.4.1. Transferred 200 mg of Tromethamine to a 10 mL volumetric flask.

7.2.4.2. Stored the sample in an oven set at 60 °C for 5 days.

7.2.4.3. Pipetted 0.6 mL of water, diluted to volume with methanol, and mixed.

7.2.5. Oxidative Sample (20 mg/mL Tromethamine)

7.2.5.1. Transferred 200 mg of Tromethamine into a 10 mL volumetric flask and pipetted 0.5 mL of water and 0.1 mL of 30% Hydrogen Peroxide into the flask.

7.2.5.2. Allowed the solution to sit for 2 days at room temperature.

7.2.5.3. Diluted to volume with methanol and mixed.

7.2.5.4. Note: Due to excessive assay impact, the oxidative stress was reduced to 50% and 25% levels to induce less degradation (1-5% target).

7.2.6. Control Sample (20 mg/mL Tromethamine)

7.2.6.1. Transferred 200 mg of Tromethamine into a 10 mL volumetric flask, pipetted 0.6 mL of water to the flask, diluted to volume with methanol, and mixed.

7.2.7. Hydrolysis Blank

7.2.7.1. Pipetted 0.3 mL of 0.1N Hydrochloric Acid and 0.3 mL of 0.1N Sodium Hydroxide to a 10 mL volumetric flask, diluted to volume with methanol and, mixed.

7.2.8. Oxidative Blank

7.2.8.1. Pipetted 0.1 mL of 30% Hydrogen Peroxide and 0.5 mL of water to a 10 mL volumetric flask, diluted to volume with methanol, and mixed.

7.3. **Solution Preparation – Accuracy, Precision, and Linearity**

7.3.1. Prepared the following concentrations of Tromethamine samples for performance analysis.

Table 7: Assay-Level Performance Samples

Concentration Level	Actual Prepared Concentration Level (mg/mL)	Actual Prepared Concentration Level (mg/mL)	Actual Prepared Concentration Level (mg/mL)
24 mg/mL	24.013	24.006	24.008
22 mg/mL	22.006	22.002	22.008
20 mg/mL	20.012	20.016	20.020
20 mg/mL	20.006	20.010	20.016
18 mg/mL	18.016	18.014	18.008
16 mg/mL	16.010	16.006	16.006

Table 8: Unspecified Impurity- Level Performance Samples

Concentration Level	Actual Prepared Concentration Level (mg/mL)	Actual Prepared Concentration Level (mg/mL)	Actual Prepared Concentration Level (mg/mL)
0.040 mg/mL	0.0406	0.0406	0.0406
0.030 mg/mL	0.0304	0.0300	0.0305
0.020 mg/mL	0.0203	0.0201	0.0202
0.020 mg/mL	0.0202	0.0204	0.2020
0.010 mg/mL	0.0101	0.0101	0.0101
0.006 mg/mL	0.0060	0.0061	0.0061

7.4. **Setting up the instrument:**

7.4.1. Set up the Shimadzu QP2010S GC-FID using the method parameters specified in section 5.6.

7.5. **Injection Sequence:**

7.5.1. Each sample was injected once with a split ratio of 75 for the assay level and a second time with a split of 5 for the impurity level.

Table 9: Injection Sequence

Sample ID	Number of Injections
System Suitability	
Diluent	≥1
LOQ	≥3
SS1	5
SS2 (Standard Check)	2
Diluent	1
Unspecified Impurity-Level Standard	5
Samples	
Samples	≤6 (1 injection each)
SS1 (QC Check)	1
Diluent	1
Unspecified Impurity-Level Standard (QC Check)	1
• Repeat the sample injection sequence if additional samples are to be analyzed • Samples may be substituted with diluent injections	

7.6. **System Suitability Criteria**

Table 10: System Suitability Criteria

System Suitability Parameter	Acceptance Criteria
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first (5) injections of the SS1 solution.	NMT 1.0%
The average %Agreement between the first five (5) SS1 injections and each SS1 (QC Check).	98% to 102%
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Unspecified Impurity-Level Standard solution.	NMT 5.0%
System Suitability Parameter	**Acceptance Criteria**
The average %Agreement between the first five (5) Unspecified Impurity-Level Standard injections and each Unspecified Impurity-Level Standard (QC Check).	96% to 104%
Average %Agreement between the first five (5) SS1 injections and the SS2 injections.	98% to 102%
The USP tailing factor of the Tromethamine peak from the first SS1 injection.	0.6 to 1.2
Signal to noise ratio for the LOQ injection.	NLT 10:1

7.7. **Processing Chromatograms:**

7.7.1. Algorithm = Chromatopac

7.7.2. Enable Peak detection = 2.1 min

7.7.3. Width = 3 sec

7.7.4. Slope = 5,000 uV/min

7.7.5. Drift = 0 uV/min

7.7.6. T.DBL = 1,000 min

7.7.7. Minimum Area/Height: 500 counts

7.8. **Calculations:**

7.8.1. Assay

$$Assay\ (\%) = \frac{r_u}{Ar_s} \times \frac{C_s}{C_u} \times 100$$

7.8.1.1. Where:

7.8.1.1.1. r_u= peak response of Tromethamine from the Sample Solution.

7.8.1.1.2. Ar_s= Average Peak response of Tromethamine from the *Standard Solution*.

7.8.1.1.3. C_s= Concentration of Tromethamine RS in the standard solution (mg/mL prepared * Purity of CRS).

7.8.1.1.4. C_u= concentration of Tromethamine in the *Sample Solution* (mg/mL).

7.8.2. Unspecified Impurities:

$$\text{Unspecified Impurities (\%)} = \frac{r_u}{Ar_s} \times \frac{C_s}{C_u} \times 100$$

7.8.2.1. Where:

7.8.2.1.1. r_u= peak response of unspecified impurity from the *Sample Solution*.

7.8.2.1.2. Ar_s= Average Peak response of Tromethamine from the Unspecified Impurity-level Standard.

7.8.2.1.3. C_s= Concentration of Tromethamine RS in the Unspecified Impurity-level Standard (mg/mL prepared * Purity of CRS).

7.8.2.1.4. C_u= concentration of Tromethamine in the *Sample Solution* (mg/mL).

8. PERFORMANCE REPORT:

8.1. System Suitability: Assay

8.1.1. Injected the Assay Standard (SS1), Assay Standard (SS2), and Assay Standard (SS1) (QC Checks) as per the Injection Sequence Table.

8.1.2. Acceptance Criteria:

8.1.2.1. Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the SS1 solution: NMT 1.0%.

8.1.2.2. The average %Agreement between the first five (5) SS1 injections and each SS1 (QC Check): 98% to 102%.

8.1.2.3. Average %Agreement between the first five (5) SS1 injections and the SS2 injections: 98% to 102%.

8.1.2.4. The USP tailing factor of the Tromethamine peak from the first SS1 injection: 0.6 – 1.2.

Table 11: System Suitability Assay Data

Replicate	Tris Retention Time (min)	Area Count	Average	% RSD (NMT 1.0%)	Result
1	5.209	1054361	1056771	0.25	Pass
2	5.209	1060103			
3	5.208	1059076			
4	5.206	1056974			
5	5.205	1053341			

Table 12: System Suitability Summary Results Assay

BSI-ATM-0111, Assay of Tromethamine Via GC-FID System Suitability Summary			
System Suitability Requirement	**Acceptance Criteria**	**Results**	**Pass/Fail**
The relative standard deviation of the Tromethamine peak from the first (5) injections of the SS1 solution.	NMT 1.0%	0.25%	Pass
The average %Agreement between the first five (5) SS1 injections and each SS1 (QC Check).	98% to 102%	QC Check 1: 100% QC Check 2: 100% QC Check 3: 100%	Pass
Average %Agreement between the first five (5) SS1 injections and the SS2 injections.	98% to 102%	100%	Pass
The USP tailing factor of the Tromethamine peak from the first SS1 injection.	0.6 to 1.2	0.757	Pass
Notebook GC06 p.28			

8.2. **System Suitability: Unspecified Impurity-Level**

8.2.1. Injected the Unspecified Impurity-Level Standard, Unspecified Impurity-Level Standard (QC Check), and the LOQ solution as per the Injection Sequence Table.

8.2.2. Acceptance Criteria:

8.2.2.1. Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Unspecified Impurity-Level Standard Solution: NMT 20%.

8.2.2.2. The average %Agreement between the first five (5) Unspecified Impurity-Level Standard injections and each Unspecified Impurity-Level Standard (QC Check): 80% to 120%.

8.2.2.3. The Signal to Noise Ratio for the LOQ injections: NLT 10:1

Table 13: System Suitability Unspecified Impurity-Level Data

Replicate	Retention Time (min)	Area Count	Average	% RSD (NMT 20%)	Result
1	5.091	134601	135322	0.65	Pass
2	5.090	135497			
3	5.090	135850			
4	5.091	136561			
5	5.092	134102			
Notebook pages: GC06 p.33-35					

Table 14: System Suitability Summary Results Unspecified Impurity Data

BSI-ATM-0112, Tromethamine Unspecified Degradation Products Via GC-FID System Suitability Summary			
System Suitability Requirement	**Acceptance Criteria**	**Results**	**Pass/Fail**
Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Unspecified Impurity-Level Standard Solution: NMT 20%.	NMT 20%	0.65%	Pass
The average %Agreement between the first five (5) Unspecified Impurity-Level Standard injections and each Unspecified Impurity-Level Standard (QC Check): 80% to 120%.	80% to 120%	QC Check 1: 100% QC Check 2: 100% QC Check 3: 99% QC Check 4: 100%	Pass
The Signal to Noise Ratio for the LOQ injections: NLT 10:1.	NLT 10:1	LOQ 1: 708 LOQ 2: 74 LOQ 3: 50	Pass
Notebook: GC06 p.34			

8.3. **Limit of Detection (LOD) / Limit of Quantitation (LOQ)**

8.3.1. **Note:** The Split Ratio was set to five (5) for these injections.

8.3.2. Injected a 0.006mg/mL Tromethamine solution as a sample as per the Injection Sequence Table six (6) times.

8.3.3. Acceptance Criteria:

8.3.3.1. Limit of Detection (LOD): The Signal to Noise Ratio for each injection is NLT 10:1.

8.3.3.2. Limit of Quantitation (LOQ): The Relative Standard Deviation (%RSD) of the Tromethamine peak areas is NMT 20%.

8.3.3.3. The LOQ level is NMT 0.03%.

8.3.4. Results

8.3.4.1. Limit of Detection (LOD): Pass

8.3.4.1.1. Average Signal to Noise Ratio = 20:1

8.3.4.2. Limit of Quantitation (LOQ): Fail

8.3.4.2.1. Relative Standard Deviation (%RSD) = 32.0%

Table 15: LOQ/LOD

Limit of Detection (LOD) / Limit of Quantitation (LOQ) Results		
Injection	**Signal to Noise Ratio**	**Peak Area**
1	16	1992
2	35	1183
3	21	1099
4	16	968
5	18	1035
6	14	1007
Average	20	1214
	%RSD	32.0%

8.4. **Linearity: Assay**

8.4.1. **Note:** The split ratio was set to seventy-five (75) for these injections.

8.4.2. Inject the 80%, 90%, 100%, 110%, and 120% Tromethamine Calibration Level Samples at least once (the average of the Accuracy and Precision samples may be utilized). Plotted the peak area response against concentration and performed a linear regression by the method of least squares. Determined the Slope, Y-Intercept, Correlation Coefficient (r^2), and Y-Intercept Bias.

8.4.3. Acceptance Criteria:

8.4.3.1. Report the Y-Intercept, Slope, and Residual Sum of Squares.

8.4.3.2. Correlation Coefficient (r^2): NLT 0.995.

8.4.3.3. Y-Intercept Bias: NMT 5.0%.

8.4.4. Result: Pass

8.4.4.1. Y-Intercept: -31669

8.4.4.2. Slope: 54640

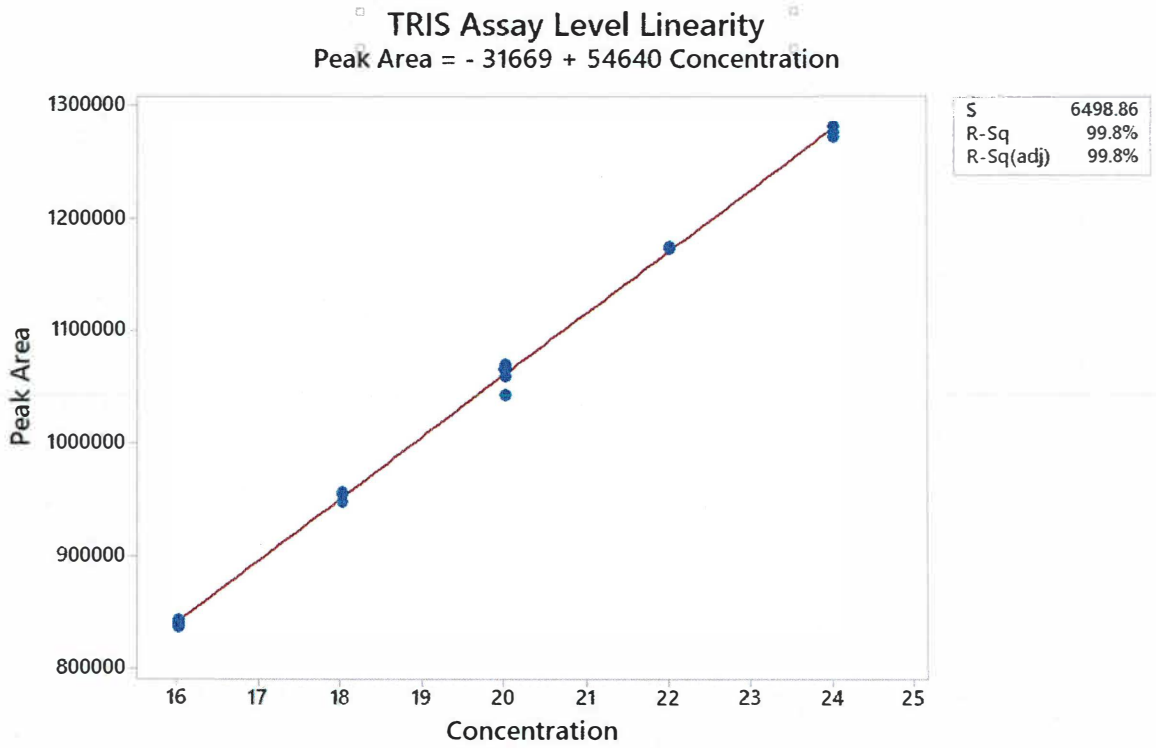
8.4.4.3. Correlation Coefficient (r^2): 0.998

8.4.4.4. Y-Intercept Bias: 2.98%

8.4.4.5. Residual Sum of Squares: 6498.86

Table 16: Linearity Assay

Level (%)	Concentration (mg/mL)	Peak Area
80	16.010	840875
80	16.006	844130
80	16.006	837748
90	18.016	956880
90	18.014	955477
90	18.008	949307
100	20.006	1066582
100	20.010	1060104
100	20.016	1042922
100	20.012	1069978
100	20.016	1065615
100	20.020	1067448
110	22.006	1175072
110	22.002	1172548
110	22.008	1173814
120	24.012	1272555
120	24.006	1282262
120	24.008	1276870



8.5. **Linearity: Unspecified Impurity-Level**

8.5.1. **Note:** The split ratio is set to five (5) for these determinations.

8.5.2. Inject the 0.03%, 0.05%, 0.10%, 0.15%, and 0.20% Tromethamine Calibration Level samples at least once (the average of the Accuracy and Precision samples may be utilized). Plotted the peak area response against concentration and perform a linear regression by method of least squares. Determined the Slope, Y-Intercept, Correlation Coefficient (r^2), and Y-Intercept Bias.

8.5.3. Acceptance Criteria:

8.5.3.1. Report the Y-Intercept, Slope and Residual Sum of Squares.

8.5.3.2. Correlation Coefficient (r^2): NLT 0.950.

8.5.3.3. Result: Pass

8.5.3.3.1. Y-Intercept: -3442

8.5.3.3.2. Slope: 676319

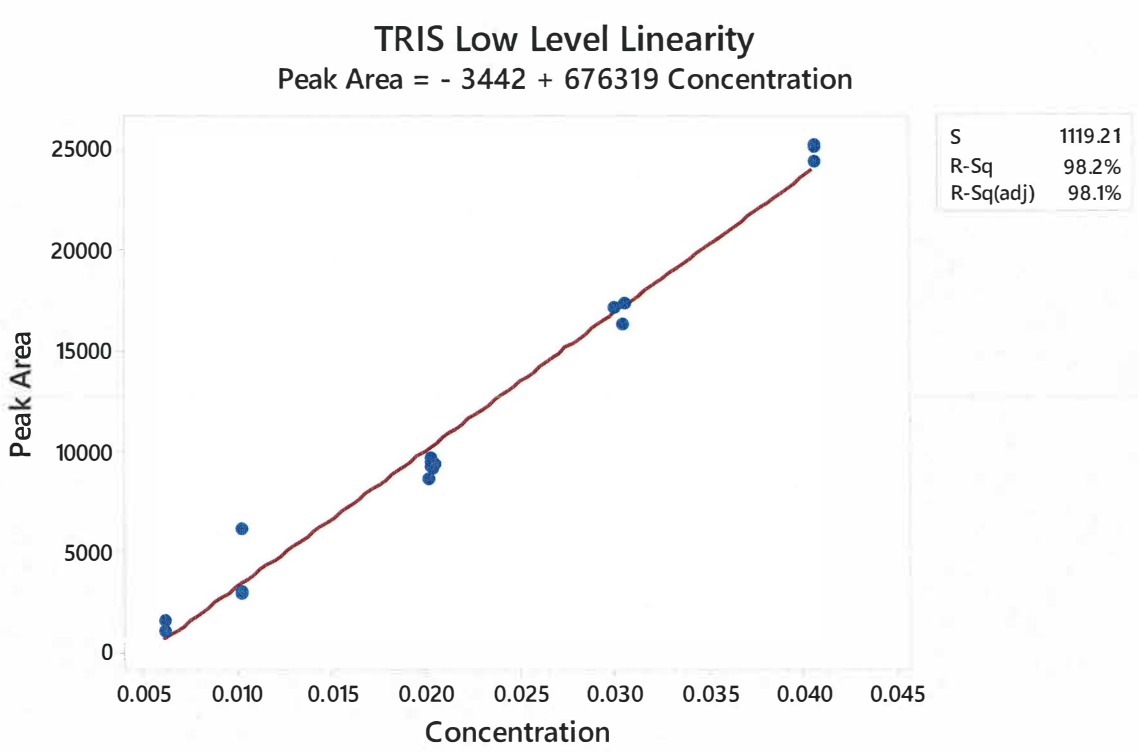
8.5.3.3.3. Correlation Coefficient (r^2): 0.982

8.5.3.3.4. Y-Intercept Bias: 37.32%

8.5.3.3.5. Residual Sum of Squares: 1119.21

Table 17: Linearity Unspecified Impurity

Level (%)	Concentration (mg/mL)	Peak Area
0.03	0.0060	1580
0.03	0.0061	1538
0.03	0.0061	1024
0.05	0.0101	2847
0.05	0.0101	2953
0.05	0.0101	6149
0.10	0.0202	9431
0.10	0.0204	9336
0.10	0.0202	9660
0.10	0.0203	9097
0.10	0.0201	8608
0.10	0.0202	9213
0.15	0.0304	16303
0.15	0.0300	17050
0.15	0.0305	17317
0.20	0.0406	24357
0.20	0.0406	25112
0.20	0.0406	25164



8.6. **Accuracy: Assay**

8.6.1. Injected each of the triplicate preparations of the 80%, 90%, 100%, 110%, 120% Tromethamine Calibration Level Samples and inject each of the six (6) preparations of the 100% Tromethamine Calibration Level Samples.

8.6.2. Acceptance Criteria:

8.6.2.1. Percent Recovery (%): All replicates are between 99% to 101%.

8.6.2.2. Comparison of results with those obtained by another well-characterized technique.

8.6.2.3. Difference of mean values ($n \geq 6$ samples) 98.0% – 102.0%: $\Delta \leq 1.0\%$ abs.

8.6.3. Result: Pass

8.6.3.1. Notebook pages: GC06/26-28 for GC Assay values and MV11/70-71 for Titration Assay values

Table 18: Accuracy: 100% Assay

Replicate	r_u	Ar_s	C_s (mg/mL)	C_u (mg/mL)
1	1066582	1056771	20.006	20.006
2	1060104			20.010
3	1042922			20.016
4	1099978			20.012
5	1065615			20.016
6	1067448			20.020

Table 19: Accuracy: Comparison

Accuracy: Assay – Comparison with Well Characterized Technique Results		
Sample	**GC Assay Value (%)**	**Titration Assay Value (%)**
1	100.9	100.0
2	100.2	100.0
3	98.6	100.0
4	101.1	99.9
5	100.7	100.0
6	100.9	99.9
Average	100.4	100.0
Difference of mean values (Δ)	0.4	

8.7. **Precision: Assay**

8.7.1. Injected each of the six (6) preparations of the 100% Tromethamine Calibration Level Samples.

8.7.2. Acceptance Criteria:

8.7.2.1. Relative Standard Deviation (%RSD): NMT 1.5%

8.7.3. Result: Pass

8.7.3.1. Notebook pages: GC06 p. 26-28

Table 20: Precision: Assay Results

Sample	Assay Result (%)
Replicate 1	100.9
Replicate 2	100.2
Replicate 3	98.6
Replicate 4	101.1
Replicate 5	100.7
Replicate 6	100.9
Average	100.4
%RSD	0.9%

8.8. **Intermediate Precision: Assay**

8.8.1. **Note:** The split ratio is set to seventy-five (75) for these determinations.

8.8.2. A different analyst or qualified designee repeated the 100% Tromethamine Calibration Level portion of the protocol. The was performed on a different day with separately prepared samples and standards.

8.8.3. Acceptance Criteria:

8.8.3.1. Difference of mean values Δ: ≤ 1.5% rel

8.8.4. Result: Pass

8.8.4.1. Notebook pages for Analyst II: MV11/75

Table 21: Intermediate Precision: Assay Results

Sample	Analyst I Assay Result (%)	Analyst II Assay Result (%)
Replicate 1	100.9	100.5
Replicate 2	100.2	101.2
Replicate 3	98.6	101.1
Replicate 4	101.1	100.6
Replicate 5	100.7	100.2
Replicate 6	100.9	101.4
Average	100.4	100.8
%RSD	0.9%	0.4%
Difference of Mean Value	0.4%	

8.9. **Precision: Unspecified Impurity - Level**

8.9.1. **Note:** The spit ratio was set to five (5) for these determinations.

8.9.2. Injected each of the six (6) preparations of the 0.10% Tromethamine Calibration Level samples.

8.9.3. Acceptance Criteria:

8.9.3.1. Relative Standard Deviation (%RSD): NMT 20%.

8.9.4. Result: Pass

8.9.4.1. Notebook Page: GC06 p. 33-35

Table 22: Precision: Unspecified Impurity-Level Results

Sample	Unspecified Impurity-Level Result (%)
Replicate 1	69.0
Replicate 2	67.8
Replicate 3	70.8
Replicate 4	66.3
Replicate 5	63.3
Replicate 6	67.6
Average	65.7
%RSD	2.8

8.10. **Accuracy/Intermediate Precision: Unspecified Impurity-Level**

8.10.1. **Note:** The split ratio was set to five (5) for these determinations.

8.10.2. A different analyst of qualified designee repeated the 0.10% Tromethamine Calibration Level portion of the protocol. This was performed on a different day with separately prepared samples and standards.

8.10.3. Acceptance Criteria:

8.10.3.1. Difference of Mean Values Δ: ≤30 rel.

8.10.4. Result: Pass

8.10.4.1. Notebook page: GC06/33-35 for Analyst; MV11/76 for Analyst II

Table 23: Intermediate Precision: Unspecified Impurity-Level Results

Sample	Analyst I Unspecified Impurity-Level Result (%)	Analyst II Unspecified Impurity-Level Result (%)
Replicate 1	69.0	75.8
Replicate 2	67.8	70.9
Replicate 3	70.8	68.1
Replicate 4	66.3	68.3
Replicate 5	63.3	67.9
Replicate 6	67.6	67.3
Average	65.7	67.9
%RSD	2.8%	0.6%
Difference of Mean Value	2.2	

8.11. **Range: Assay**

8.11.1. The range of an analytical procedure is the interval between the upper and lower levels of analyte in the sample that have demonstrated suitable Accuracy, Precision, and Linearity.

8.11.2. Acceptance Criteria:

8.11.2.1. Report the range of the analysis from the lowest level of analyte to the highest level of analyte that meets requirements for Accuracy, Precision, and Linearity.

8.11.3. Result:

8.11.3.1. The quantitative range of the method is 16 mg/mL to 24 mg/mL of tromethamine in 6% water in methanol. Samples should be diluted to the working range of the instrumental method.

8.12. **Solution Stability: Assay Level**

8.12.1. **Note:** The split ratio was set to seventy-five (75) for these determinations.

8.12.2. Saved and re-injected an Assay Standard (SS2) after 2 days, 3 days, and 7 days.

8.12.3. Acceptance Criteria:

8.12.3.1. %Agreement between the first five (5) injections of a freshly prepared Assay Standard (SS1) and the aged Assay Standard (SS2) is 98.0% – 102.0%.

8.12.3.2. Result: Pass 3 Days

Table 24: Solution Stability: Assay Level Results

Initial Result	Day 2 Peak Area	% Agreement	Day 3 Peak Area	% Agreement	Day 7 Peak Area	% Agreement
Fresh Standard	1035552.2	0.94	1037940.6	0.04	1016616.8	2.69
Standard	1045519		1036888		1043532	

8.13. **Solution Stability: Unspecified Impurity-Level**

8.13.1. **Note:** The split ratio was set to five (5) for these determinations.

8.13.2. Save and re-inject an Impurity-level Standard after 2 day, 3 days, and 7 days

8.13.3. Acceptance Criteria:

8.13.3.1. Impurity-level: 0.03% ≤ Level < 0.15%: ≤ 30% rel.

8.13.3.2. Result: Pass 7 Days.

Table 25: Solution Stability: Unspecified Impurity-Level Results

Initial Result	Day 2 Peak Area	% Agreement	Day 3 Peak Area	% Agreement	Day 7 Peak Area	% Agreement
Fresh Standard	135021	6.43	145780	11.9	122711	8.1
Standard	143730		128340		132599	

8.14. **Specificity: Unspecified Impurity-Level**

8.14.1. Analyzed acidic, basic, photolytic, thermal, and oxidative stress samples as well as a control, hydrolysis blank, oxidative blank, and diluent.

8.14.2. Acceptance Criteria:

8.14.2.1. The analyte is sufficiently separated from other impurities and from the drug substance, no peak is interfering with the analyte peak. Retention / migration time and relative retention time of the analyte(s) are reported. Peak resolution for critical peak pairs is reported.

8.14.3. Result: Pass

8.14.3.1. All peaks were resolved from the TRIS main peak. The main degradation product peak was a peak at RRT 0.94 which was observed in all samples except for the acid hydrolysis sample where this peak was below the detection limit. The resolution from the TRIS peak was more than 1.5.

Table 26: List of Unspecified Impurities Above 300ppm (0.03%)

Sample	TRIS % Found	RRT 0.41	RRT 0.48	RRT 0.76	RRT 0.77	RRT 0.79	RRT 0.82	RRT 0.94	RRT 1.42	RRT 1.65	RRT 1.87	RRT 1.97	RRT 2.01	Total Impurities
Acid Hydrolysis	100.6	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Basic Hydrolysis	98.9	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.03
Photolytic	99.4	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.05	<0.03	<0.03	<0.03	<0.03	<0.03	0.05
Thermal	99.6	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.04	<0.03	<0.03	<0.03	<0.03	<0.03	0.04
Oxidative 50%	91.9	0.16	0.05	0.35	0.14	0.16	0.11	0.85	0.15	0.10	0.05	0.10	1.22	3.23
Oxidative 25%	93.1	0.16	0.06	0.18	<0.03	0.06	<0.03	0.13	0.06	0.07	0.04	0.07	1.00	1.83

Table 27: Mass Balance Results

Sample	TRIS Peak Area	% Found	Total Impurities (%)	Mass Balance (%)
Acid Hydrolysis	1040242	100.6	0.00	100.6
Basic Hydrolysis	1022553	98.9	0.03	99.0
Photolytic	1057594	99.4	0.05	99.5
Thermal	1060791	99.6	0.04	99.7
Oxidative 50%	950885	91.9	3.23	95.1
Oxidative 25%	968648	93.1	1.83	94.9

8.15. **Robustness: Assay**

8.15.1. **Note:** The split ratio is set to seventy-five (75) for these determinations.

8.15.2. Prepared System Suitability Solutions as per the "Solution Preparation – System Suitability Solutions" section. Evaluated each robustness condition in the table below:

Table 28: Robustness: Assay Conditions

Criteria	Low	Target	High
Initial Oven Temperature	145 °C	150 °C	155 °C
Heating Rate	8 °C/min	10 °C/min	12 °C/min
Column Head Pressure	22 kPa	25 kPa	28 kPa

8.15.3. Acceptance Criteria:

8.15.4. All system suitability parameters are met

8.15.5. Result: Pass

8.15.5.1. Notebook Pages: GC06 p. 42-46

Table 29: Robustness – Low Results

System Suitability Parameter	Acceptance Criteria	Results
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first (5) injections of the SS1 solution.	NMT 1.0%	0.25%
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Impurity-Level Assay Standard solution.	NMT 5.0%	0.93%
Average %Agreement between the first five (5) SS1 injections and the SS2 injections.	98% to 102%	101%
The USP tailing factor of the Tromethamine peak from the first SS1 injection.	0.6 to 1.2	0.747
Signal to noise ratio for the LOQ injection.	NLT 10:1	Replicate 1 = 23 Replicate 2 = 83 Replicate 3 = 37

Table 30: Robustness – Target Results

System Suitability Parameter	Acceptance Criteria	Results
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first (5) injections of the SS1 solution.	NMT 1.0%	0.81%
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Unspecified Impurity-Level Standard solution.	NMT 5.0%	0.58%
Average %Agreement between the first five (5) SS1 injections and the SS2 injections.	98% to 102%	101%
The USP tailing factor of the Tromethamine peak from the first SS1 injection.	0.6 to 1.2	0.761
Signal to noise ratio for the LOQ injection.	NLT 10:1	Replicate 1 = 155 Replicate 2 = 107 Replicate 3 = 62

Table 31: Robustness – High Results

System Suitability Parameter	Acceptance Criteria	Results
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first (5) injections of the SS1 solution.	NMT 1.0%	0.13%
The Relative Standard Deviation (%RSD) of the Tromethamine peak from the first five (5) injections of the Unspecified Impurity-Level Standard solution.	NMT 5.0%	0.46%
Average %Agreement between the first five (5) SS1 injections and the SS2 injections.	98% to 102%	101%
The USP tailing factor of the Tromethamine peak from the first SS1 injection.	0.6 to 1.2	0.774
Signal to noise ratio for the LOQ injection.	NLT 10:1	Replicate 1 = 84 Replicate 2 = 102 Replicate 3 = 78

8.16. Example Chromatograms

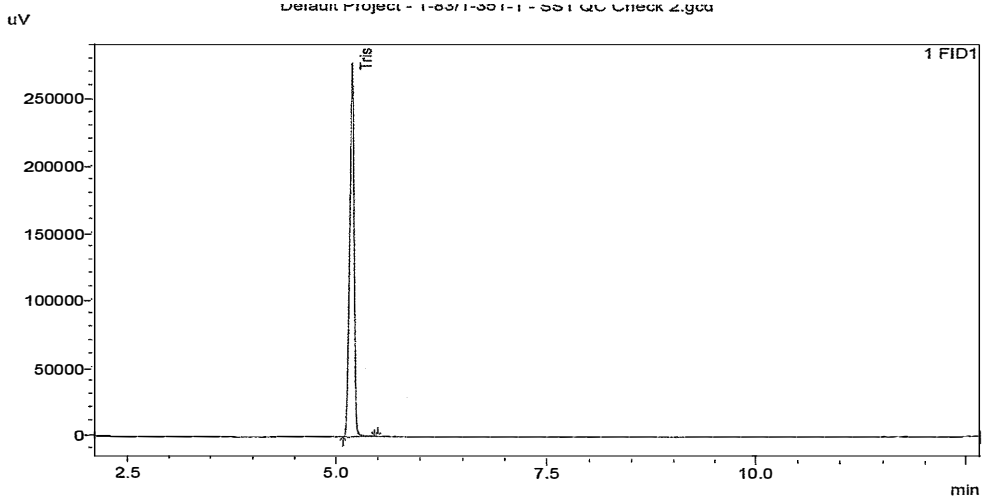


Figure 1: TRIS Assay Level Chromatogram

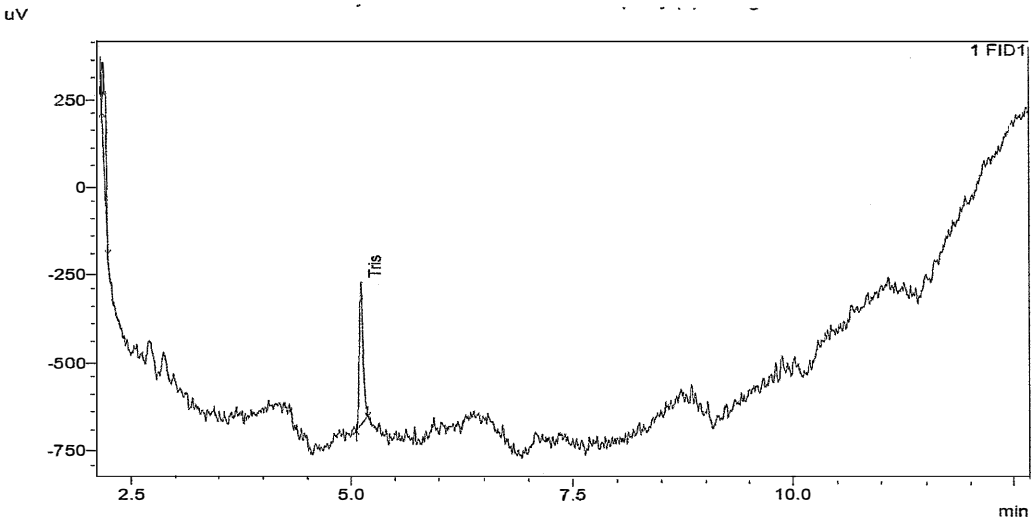


Figure 2: TRIS 0.03% Impurity LOQ Chromatogram

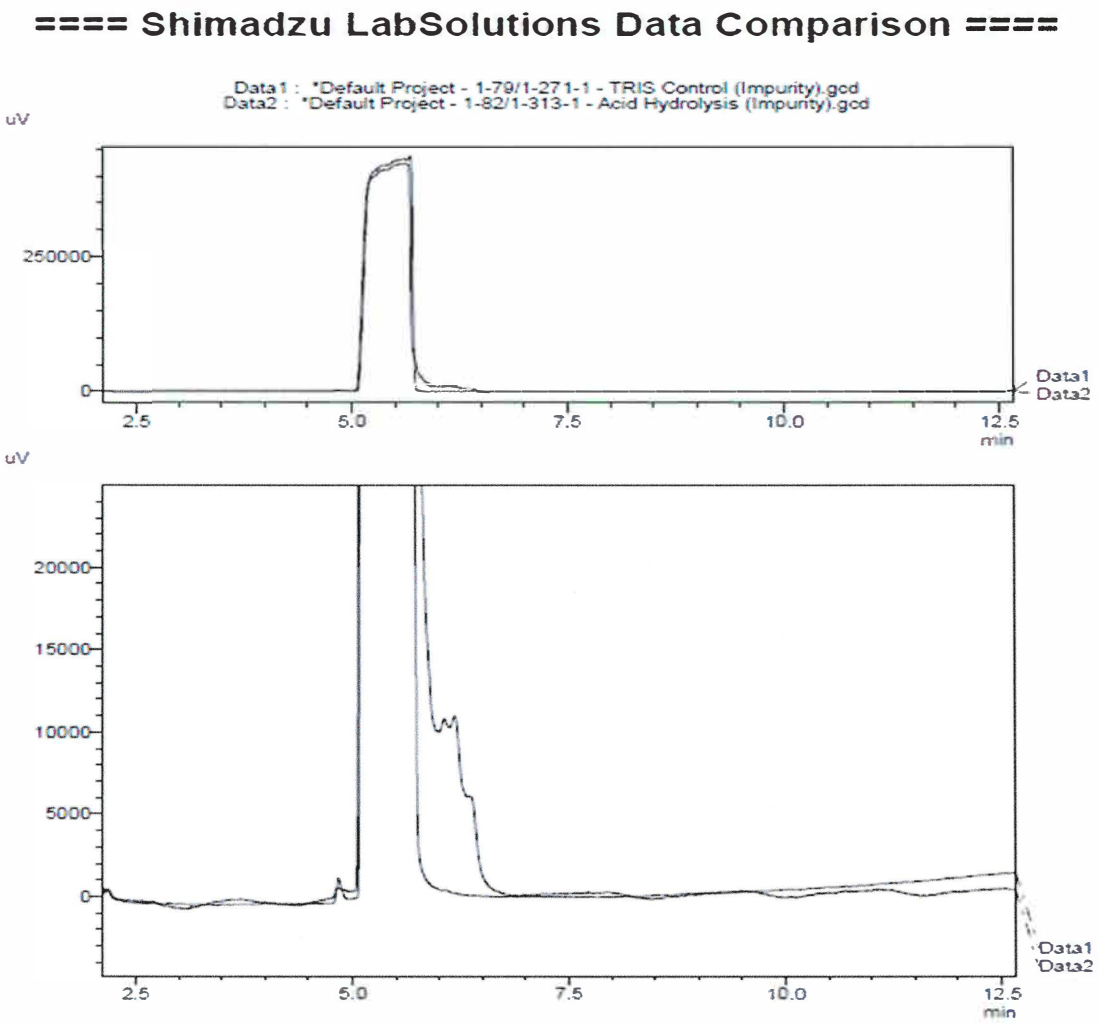


Figure 3: TRIS Acid Hydrolysis Chromatograms

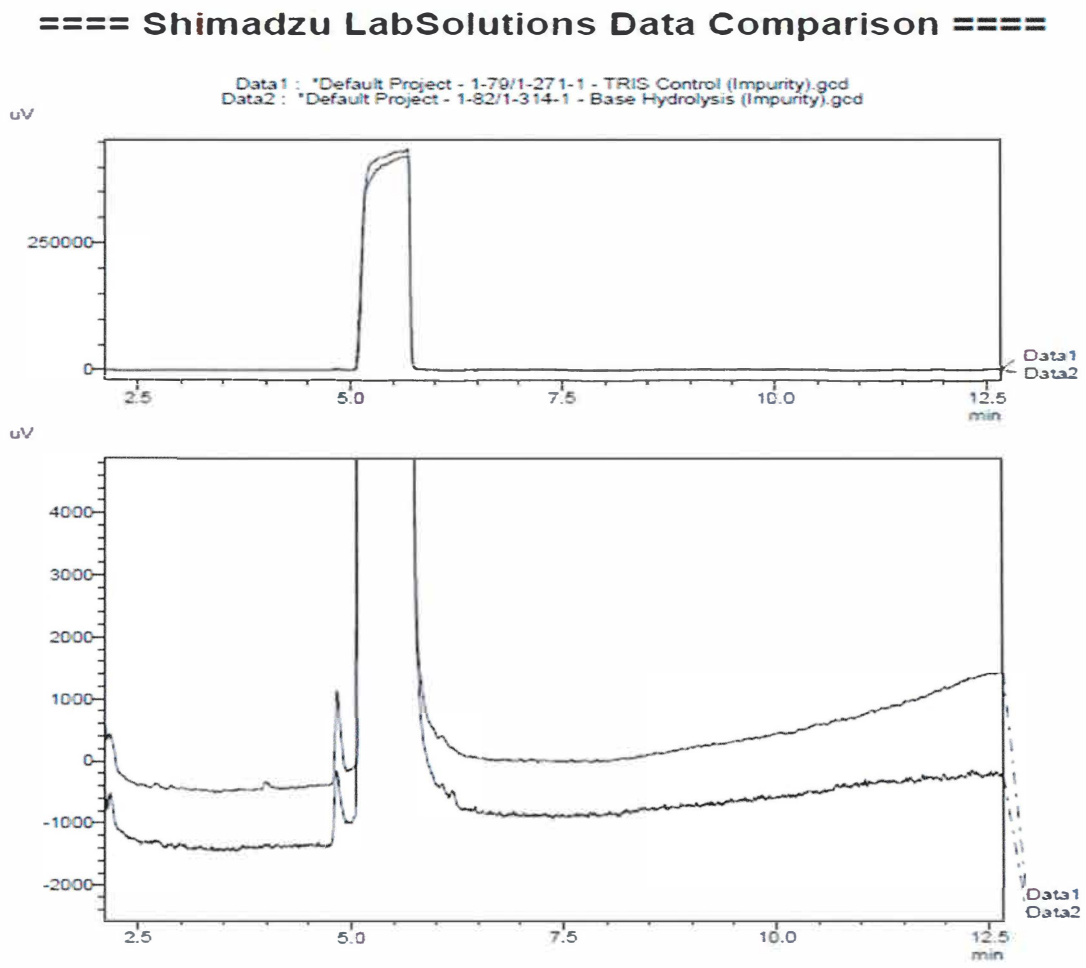


Figure 4: TRIS Base Hydrolysis Chromatograms

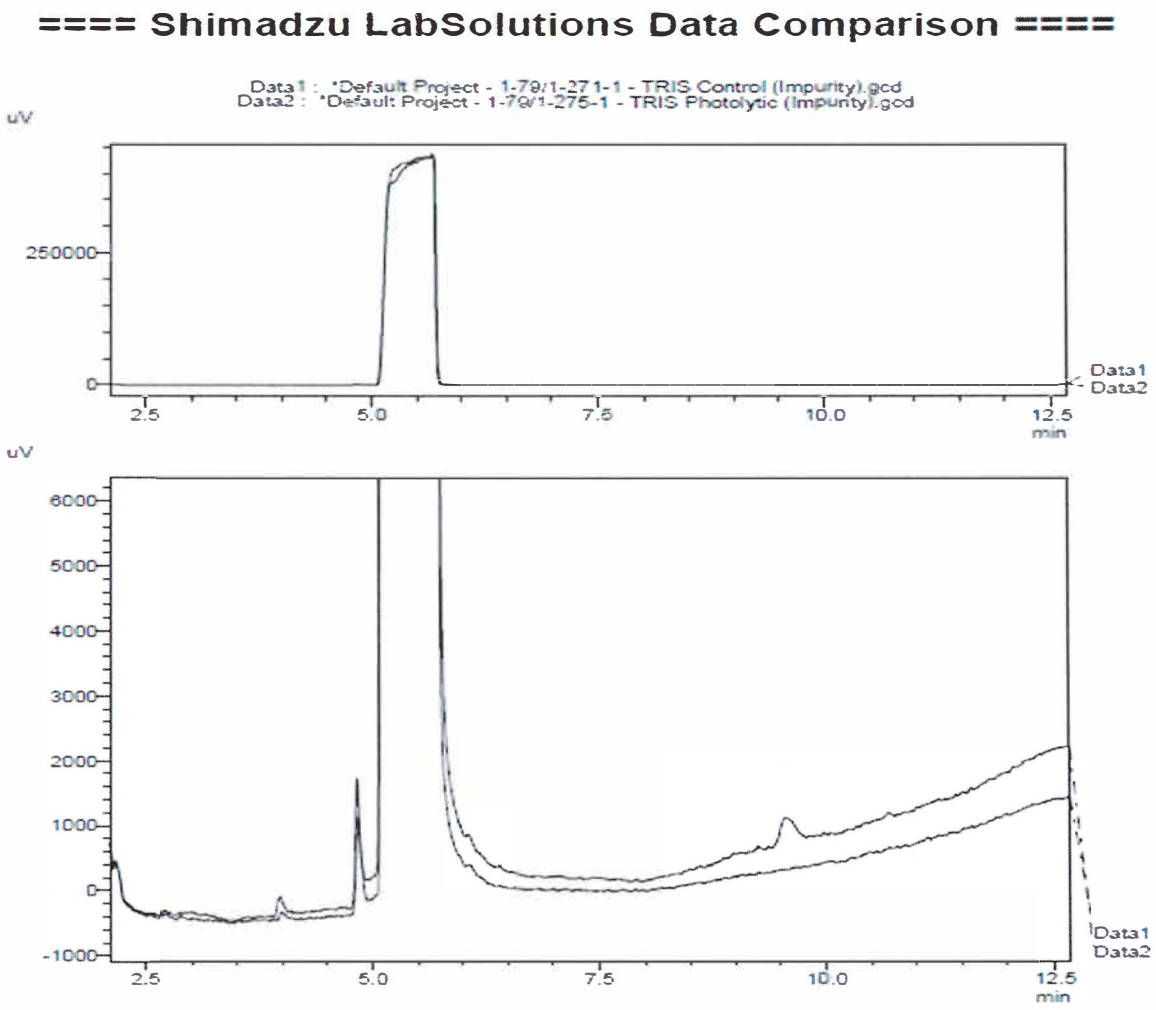


Figure 5: TRIS Photolytic Chromatograms

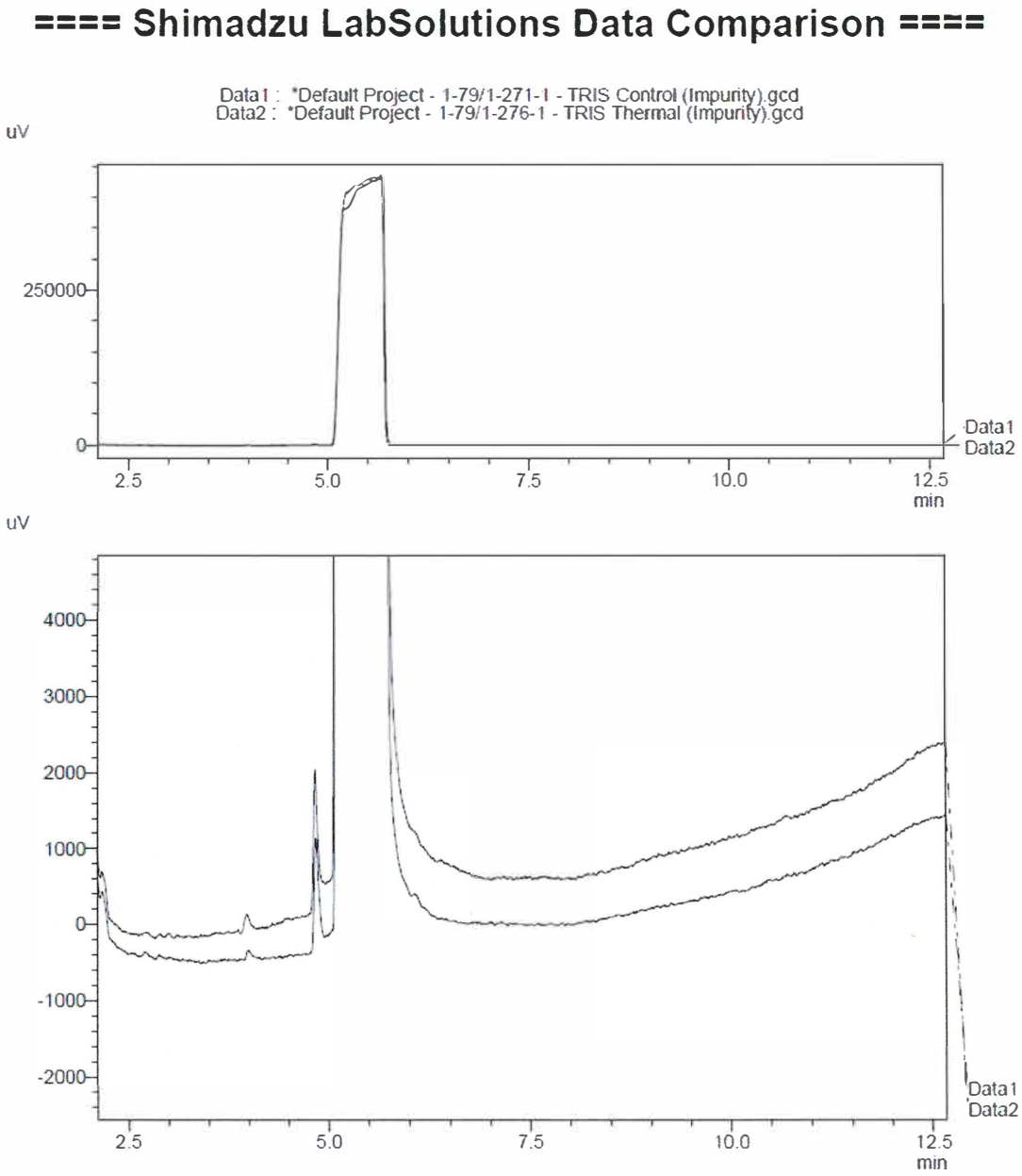


Figure 6: TRIS Thermal Chromatograms

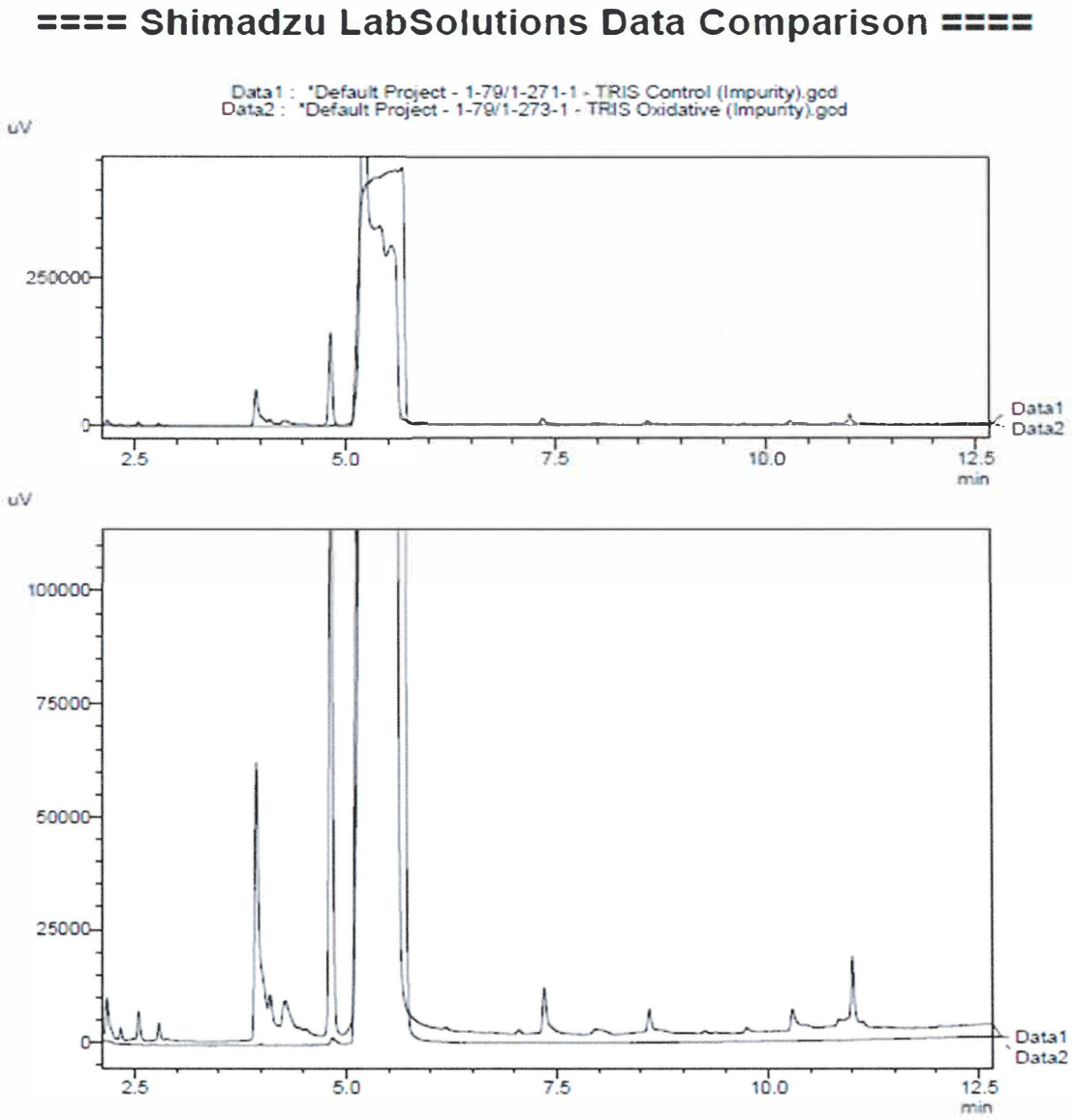


Figure 7: TRIS Oxidative Chromatograms

9. CONCLUSION:

9.1. Tromethamine Assay and Degradation product Method Validation

9.1.1. In conclusion, the Tromethamine Assay and Degradation product method via GC-FID has been adequately evaluated and validated at the BioSpectra Bangor PA facility at 100 Majestic Way. This method meets all requirements for System Suitability, Accuracy, Precision, Specificity, Linearity, Solution Stability, and Range for Assay validated as a category I. In addition, as an unspecified impurity test method, this method is considered a validated category II limit test after demonstrating sufficient detection limits (0.03% or 300 ppm) and specificity for Tris. Limit of detection of applicable limit standard must provide a signal to noise ratio of at least 3:1 based on ICH Q2 (R1) requirements for impurity testing.

9.2. Deviations from the Validation Protocol

9.2.1. **System Suitability -** All system suitability requirements were met for every run during the validation except for the oxidative, photolytic, and thermal stress sample analysis the QC Check did not meet 99-101% recovery. It was 98%. This was possibly due to the peroxide sample being injected onto the GC. All other runs met the system suitability criteria for the QC check. The results will be accepted since the run was used for mass balance calculations only on the stressed samples. This is unrelated to any Finished Good or Stability Testing Release.

9.2.2. **Limit of Quantitation (LOQ) -** The LOQ for the test method was originally set at 0.02 mg/ml in the validation protocol. However, when performing the analysis, the LOD for the method was established to be 0.006 mg/mL. The S/N ratios were all above 10:1 with an average of 20 for the 6 analyses at the LOD level of 0.006 mg/mL. The LOQ at this level did not meet the predetermined % RSD acceptance criteria of $\leq 20\%$. However, going forward for this method only the LOD will be used to determine if the specification of not more than 300 ppm will be met.

9.2.3. **Accuracy: Unspecified Impurity-Level -** The recoveries for the low-level samples were 66% for the 0.1% level samples as seen in Section 8.10.4, which did not meet quantitative validation parameters. It is theorized that some decomposition of the TRIS might be occurring in the injector. The reproducibility for the low-level samples however is quite good. Since the method itself is being used to see if there is any degradation occurring above the 0.03% level, it was decided to utilize the method as a limit test which will specify if any peaks observed are above the LOQ of 0.03% it would not meet the specification. Any detection of any unspecified impurity would lead to a batch failure.

9.2.4. **Mass Balance** - Mass balance between all stressed conditions was achieved, for the oxidative sample the mass balance as 94.9% for the Oxidative ¼ strength and 95.1 for the Oxidative ½ strength. As was stated in Section 9.2.1, the peroxide injections caused the GC produce unreproducible injections. Even with this issue a mass balance of 95% was achieved and was deemed acceptable.