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

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1. PURPOSE:

1.1. The purpose of this report is to:

1.1.1. Ensure that the Tromethamine unspecified degradation products determination via GC-FID procedure is adequately evaluated and validated.

1.1.2. Provide proof that the Tromethamine 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 unspecified degradation products determination via GC-FID method validation protocol.

2.2. This degradation method will be considered as a Category II quantitative test.

2.2.1. Assay was only included in the protocol and execution of the stress study to achieve Mass Balance. The Assay method for routine and stability analysis is performed via titration. Assay via GC-FID will not be utilized outside of the scope of this report.

2.2.2. 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.

2.2.3. 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-PRL-0688, Analytical Method Validation Protocol: Tromethamine Assay and Degradation Products via GC-FID

4.2. BSI-SOP-0098, Balance SOP

4.3. BSI-SOP-0126, Laboratory Notebooks

4.4. BSI-SOP-0134, Pipette SOP
4.5. BSI-SOP-0244, VWR Gravity Convection Operation and Calibration (Model Number: 414005-106)
4.6. BSI-SOP-0316, Shimadzu QP2010S GC/MS SOP
4.7. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.8. ICH Guideline for Analytical Validation Q2 (R1) and Q2 (R2)
4.9. ICH Guidelines for Impurities in New Drug Substances Q3A
4.10. 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:

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 Balance

Manufacturer	Model	Serial Number	Next Due	Last Service
Sartorius	MSE224S	36707108	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. Micropipette

Manufacturer	Model	Serial Number	Next Due	Last Service
Eppendorf	Research Plus	O39512B	6/30/23	12/22/22
Eppendorf	Research Plus	K53394I	6/30/23	12/22/22
Eppendorf	Research Plus	I45595H	11/30/23	5/23/23
Eppendorf	Research Plus	Q28940G	7/31/23	1/17/23
Eppendorf	Research Plus	L21310F	7/31/23	1/17/23
Eppendorf	Research Plus	J18397D	8/31/23	2/22/23
Eppendorf	Research Plus	G26211D	11/30/23	5/23/23
Eppendorf	Research Plus	R14419C	8/31/23	2/23/23

6.1.3. Equipment

Manufacturer	Model	Serial Number	Next Due	Last Service
Shimadzu	GC2010	020385050364	9/2023	9/13/22
VWR	Convection Oven	1100001176D009	8/31/23	5/23/23

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

Name	Supplier	Part No.	Lot	Due Date/Expiry	Open Date
Water	Milli-Q	Type 1 Ultra-Pure	F9SA14284H	12/31/23	Not Applicable
30% H_2O_2	Fisher	H325-500	226589	02/28/25	04/28/23
0.1N HCl	Fisher	SA54-4	231566	04/2025	05/23/23
0.1N NaOH	Fisher	SS276-4	226735	01/2025	05/12/23
Methanol	JT Baker	9093-03	22A2862003	01/18/27	06/29/23; 08/23/22

6.3. Reference Standards

Name	Supplier	Part No.	Lot	Expiry	Open Date
Tris	NIST	723e	723e	12/28/23	01/31/23

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:

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.

7.1.3. 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 Impurity-level Assay Standard Solution

7.1.4. LOQ Solution (0.02 mg/mL Tromethamine)

7.1.4.1. Pipetted 5 mL of the Impurity-level Assay 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. Specificity Solutions (Forced Degradation):

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.

7.2.9. Accuracy/Precision and Linearity Samples:

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

TABLE 1: 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 2: 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

7.3. Setting up the instrument:

7.3.1. Set up the Shimadzu QP2010S GC-MS using the method parameters specified in section 5.6.

7.4. Processing chromatograms:

7.4.1. Algorithm= Chromatopac

7.4.2. Enable Peak detection = 2.1 min

7.4.3. Width = 3 sec

7.4.4. Slope = 5,000 uV/min

7.4.5. Drift = 0 uV/min

7.4.6. T.DBL = 1,000 min

7.4.7. Minimum Area/Height: 500 counts

7.5. Calculations:

7.5.1. Assay

7.5.1.1. Result = (ru/Ars) (Cs/Cu) (100)

7.5.1.2. Where:

7.5.1.3. ru= peak response of Tromethamine from the Sample Solution.

7.5.1.4. Ars= Average Peak response of Tromethamine from the *Standard Solution.*

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

7.5.1.6. Cu= concentration of Tromethamine in the *Sample Solution* (mg/mL).

7.6. Unspecified Impurities:

7.6.1. Result = (r_u/Ar_s) (C_s/C_u) (100)

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

7.6.1.2. Ars= Average Peak response of Tromethamine from the Impurity-level Assay Standard.

7.6.1.3. Cs= Concentration of Tromethamine RS in the Impurity-level Assay Standard (mg/mL prepared * Purity of CRS).

7.6.1.4. Cu= concentration of Tromethamine in the *Sample Solution* (mg/mL).

8. PERFORMANCE REPORT:

8.1. System Suitability: Assay

8.1.1. Injected the Assay Standard Solution five times.

8.1.1.1. Acceptance Criteria:

8.1.1.1.1. Relative Standard Deviation: NMT 1.0%.

8.1.1.1.2. Result: Pass

8.1.1.1.3. Notebook pages: GC06/26-28

System Suitability 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			

8.2. System Suitability: Impurity-Level Assay

8.2.1. Injected the Impurity Level Standard Solution five times.

8.2.1.1. Acceptance Criteria:

8.2.1.1.1. Relative Standard Deviation: NMT 5.0%.

8.2.1.1.2. Result: Pass

System Suitability Data					
Replicate	**Retention Time (min)**	**Area Count**	**Average**	**% RSD (NMT 5.0%)**	**Result**
1	5.091	134601	135322	0.7	Pass
2	5.090	135497			
3	5.090	135850			
4	5.091	136561			
5	5.092	134102			
Notebook pages: GC06/33-35					

8.3. Linearity: Assay Level (**Note: the split ratio is set to 75 for these determinations**)

8.3.1. Inject the 80%, 90%, 100%, 110%, and 120% Level Solutions

8.3.1.1. Acceptance Criteria:

8.3.1.2. Report the y intercept, slope, and residual sum of squares.

8.3.1.3. Correlation coefficient ≥ 0.995

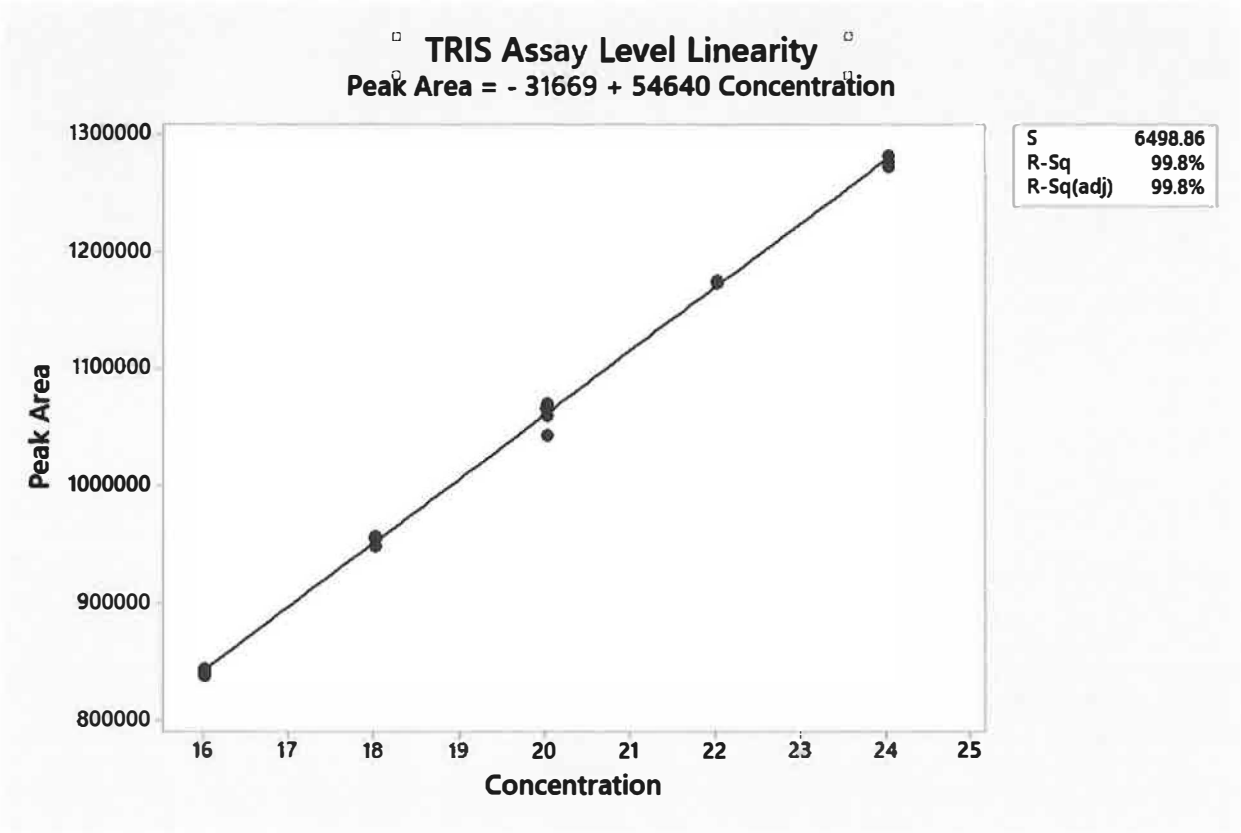
8.3.1.4. Result: Pass

8.3.1.5. Y-Intercept: -31669

8.3.1.6. Slope: 54640

8.3.1.7. Residual Sum of Squares: 6498.86

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



8.4. Linearity: Impurity Level (**Note: the split ratio is set to 5 for these determinations**)

8.4.1. Inject the 0.03%, 0.05%, 0.10%, 0.15%, and 0.20% Level Solutions

8.4.1.1. Acceptance Criteria:

8.4.1.1.1. Report the y intercept, slope and residual sum of squares.

8.4.1.1.2. Correlation coefficient ≥ 0.950

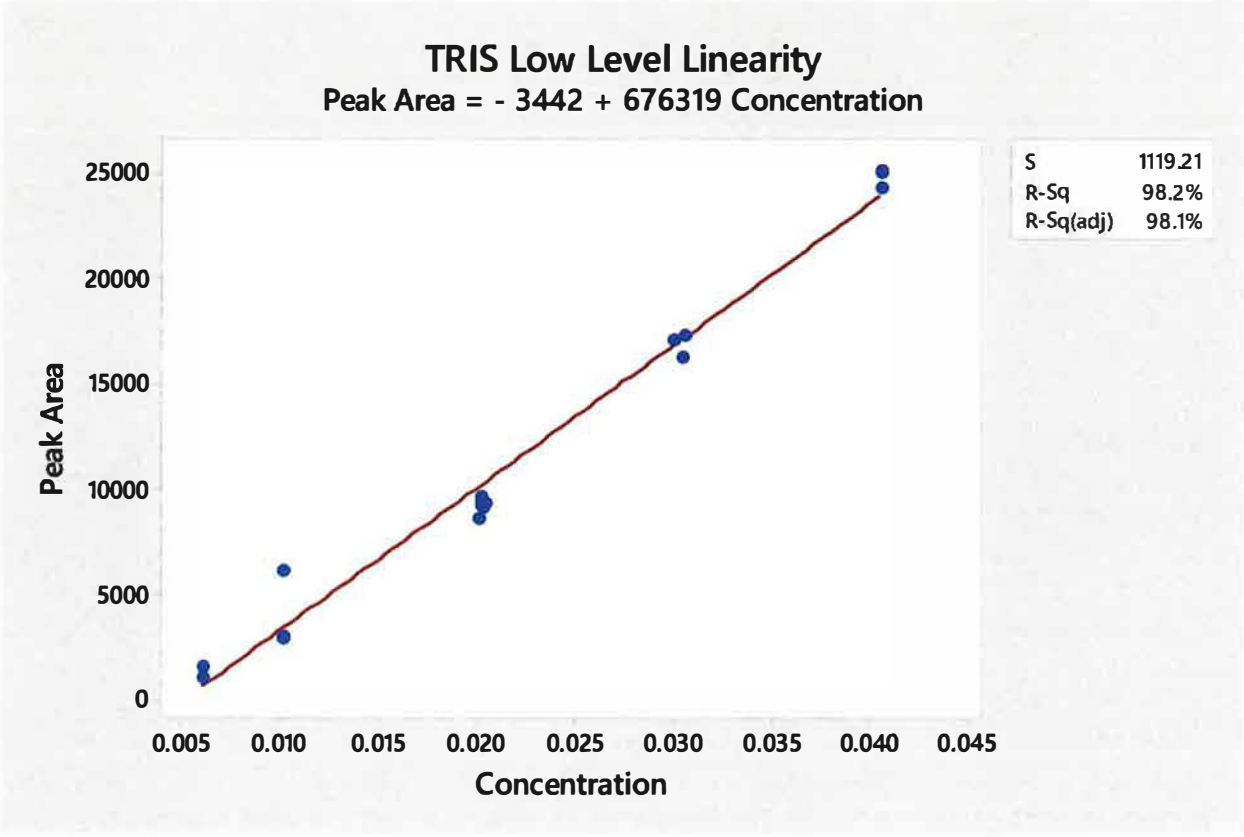
8.4.1.1.3. Result: Pass

8.4.1.1.4. Y-Intercept: -3442

8.4.1.1.5. Slope: 676319

8.4.1.1.6. Residual Sum of Squares:1119.21

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



8.5. Accuracy

8.5.1. Compare the Assay results to results obtained by Titration

8.5.1.1. Acceptance Criteria:

8.5.1.1.1. Difference of mean values (n ≥ 6 samples) 98.0 – 102.0%: Δ ≤ 1.0% abs

8.5.1.1.2. Δ: 0.51

8.5.1.1.3. Result: Pass

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

8.6. Assay Result = (r_u/Ar_s) (C_s/C_u) (100)

8.6.1. Where:

8.6.2. ru= peak response of Tromethamine from the Sample Solution.

8.6.3. Ars= Average Peak response of Tromethamine from the Standard Solution.

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

8.6.5. Cu= concentration of Tromethamine in the *Sample Solution* (mg/mL)

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

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	0.4	

8.7. Precision and Intermediate Precision: Assay Level (**Note: the split ratio is set to 75 for these determinations**)

8.7.1. Precision: Inject 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: ≤ 1.5% (n ≥ 6).

8.7.2.2. Result: Pass

8.7.3. Intermediate Precision: A different analyst or qualified designee will repeat the 100% Tromethamine Calibration Level portion of Section

8.7.4. Acceptance Criteria:

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

8.7.4.2. Result: Pass

8.7.4.3. Notebook pages for Analyst II: MV11/75

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

8.8. Precision and Intermediate Precision: Impurity Level (**Note: the split ratio is set to 5 for these determinations**)

8.8.1. Precision: Inject each of the six (6) preparations of the 0.10% Tromethamine Calibration Level samples.

8.8.2. Acceptance Criteria:

8.8.2.1. Difference of mean values Δ: 30% rel.

8.8.2.2. Result: Pass

8.8.3. Intermediate Precision: A different analyst or qualified designee will repeat the 100% Tromethamine Calibration Level portion of Section

8.8.4. Acceptance Criteria:

8.8.4.1. Difference of mean values Δ: ≤ 30 rel

8.8.4.2. Result: Pass

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

Sample	Result % Recovery Analyst 1	Result % Recovery Analyst II
1	69.0	75.8
2	67.8	70.9
3	70.8	68.1
4	66.3	68.3
5	63.3	67.9
6	67.6	67.3
Average	65.7	67.9
RSD	2.8	0.6
Difference in Mean Value	2.2	

8.9. LOD/LOQ Assessment: (**Note: the split ratio is set to 5 for these determinations**)

8.9.1. Injected the 0.006 mg/mL solution as a sample as per Section 8.6 six (6) times.

8.9.2. Acceptance criteria:

8.9.2.1. The relative standard deviation of the tromethamine peak areas is NMT 20% to meet LOQ requirements.

8.9.2.2. The S/N ratio of NLT 10 is required to meet LOD requirements.

8.9.2.3. Result: Pass, LOD; Fail, LOQ

Injection	Peak Area (LOD)	S/N Ratio
1	1992	16
2	1183	35
3	1099	21
4	968	16
5	1035	18
6	1007	14
Average	1214	20
% RSD	32.0	

8.10. Range Assay

8.10.1. The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written.

8.10.2. 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.

8.11. Solution Stability Assay Level (**Note: the split ratio is set to 75 for these determinations**)

8.11.1. Save and re-inject an Assay Standard (SS2) after 2 days, 3 days, and 7 days.

8.11.2. Acceptance criteria:

8.11.2.1. %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%.

8.11.2.2. Result: Pass 3 Days

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

8.12. Solution stability Impurity Level (**Note: the split ratio is set to 5 for these determinations**)

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

8.12.2. Acceptance criteria:

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

8.12.2.2. Result: Pass 7 Days

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

8.13. Specificity: Impurity-level

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

8.13.2. Acceptance criteria: 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.

8.13.3. 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.

8.13.4. Result: Pass Specificity

List of 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

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

8.14. Robustness (**Note: the split ratio is set to 75 for these determinations**)

8.14.1. Acceptance criteria

8.14.2. All system suitability parameters are met

8.14.3. Result: Pass

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. Example chromatograms:

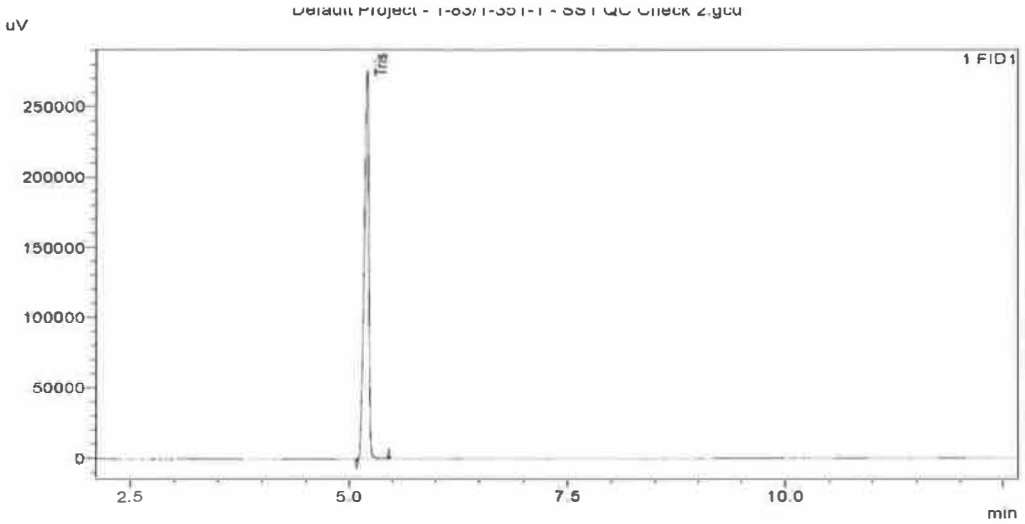


Figure 1: TRIS Assay Level Chromatogram

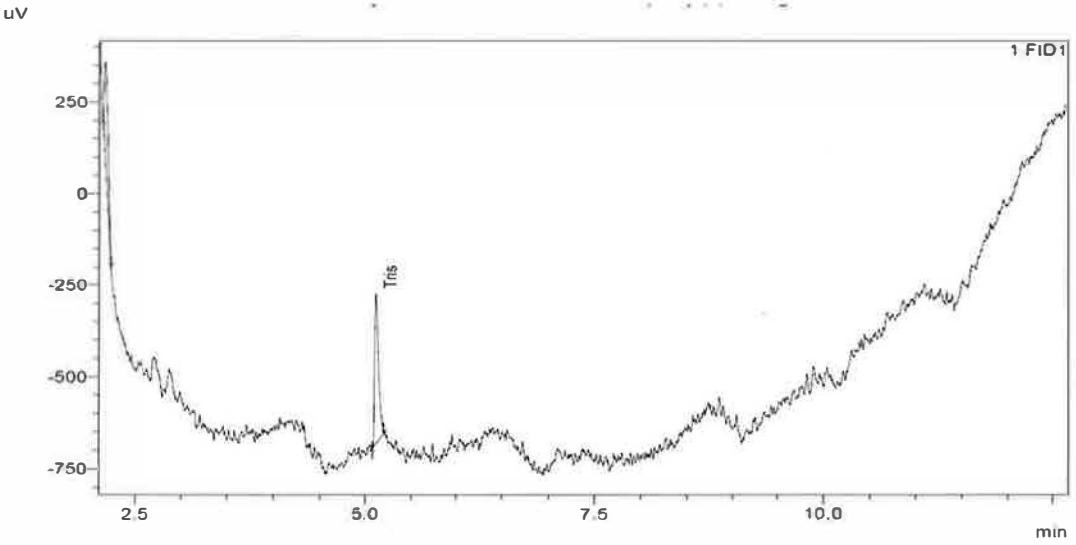


Figure 2: TRIS 0.03% Impurity LOQ Chromatogram

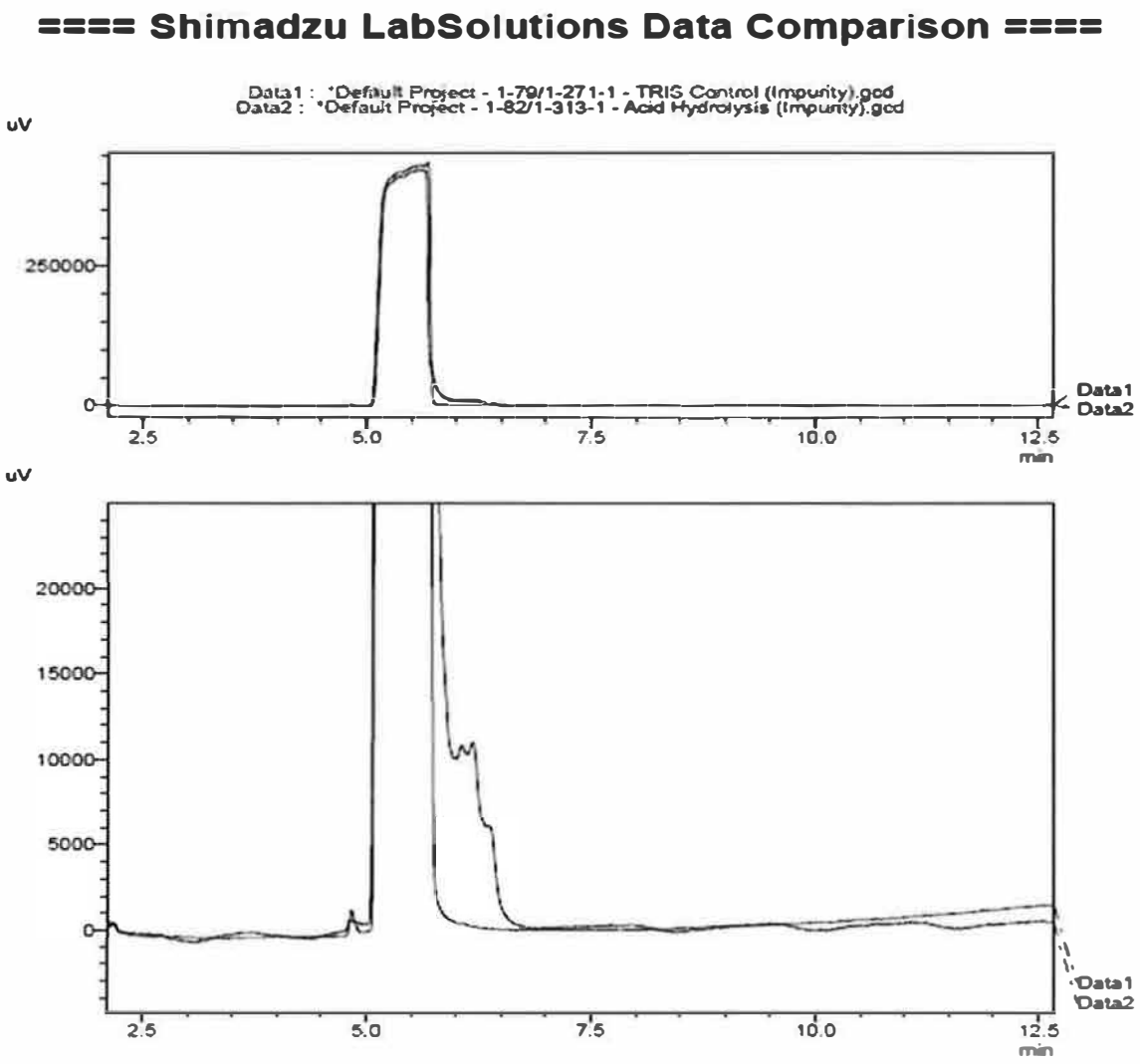


Figure 3: TRIS Acid Hydrolysis Chromatograms

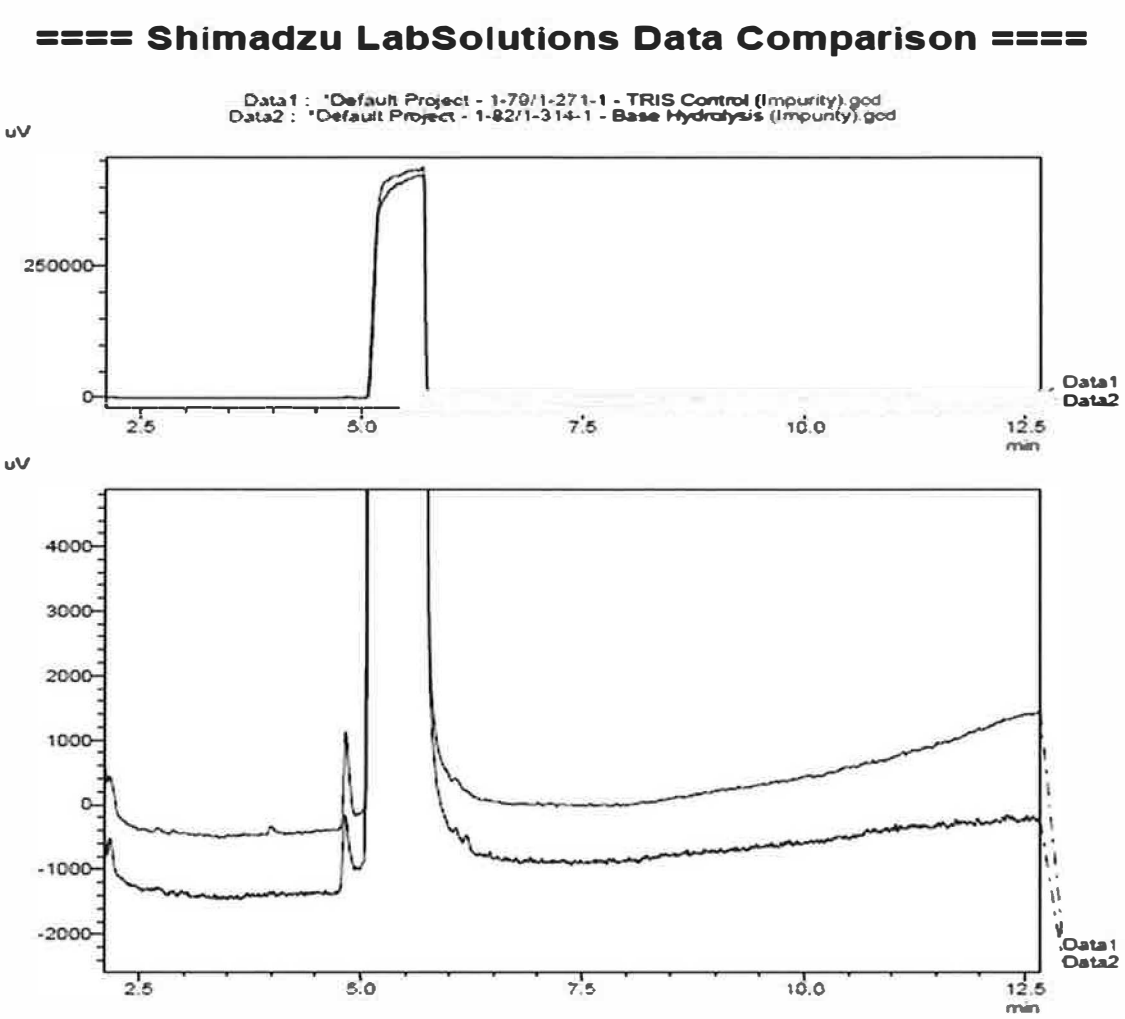


Figure 4: TRIS Base Hydrolysis Chromatograms

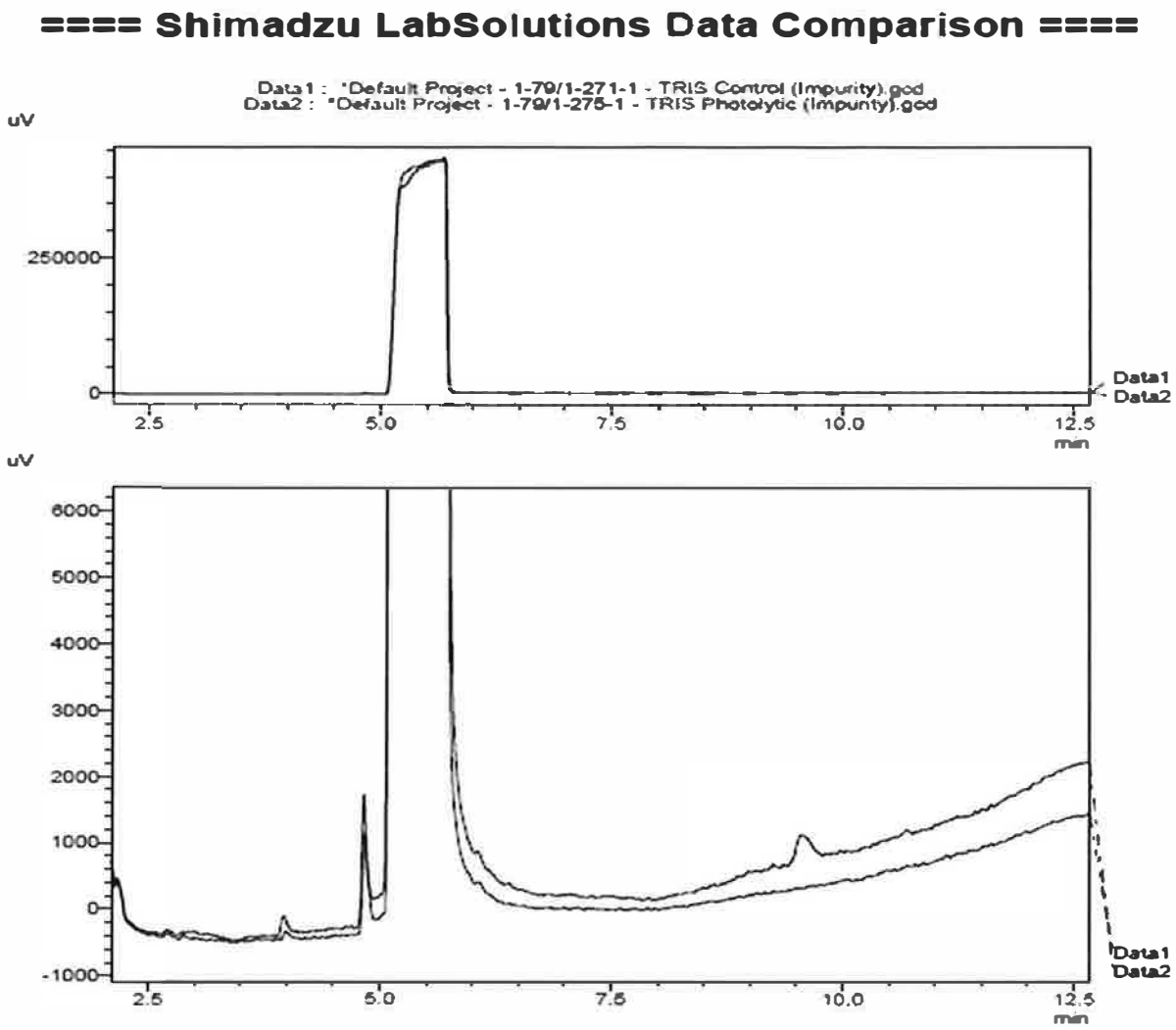


Figure 5: TRIS Photolytic Chromatograms

==== Shimadzu LabSolutions Data Comparison ====

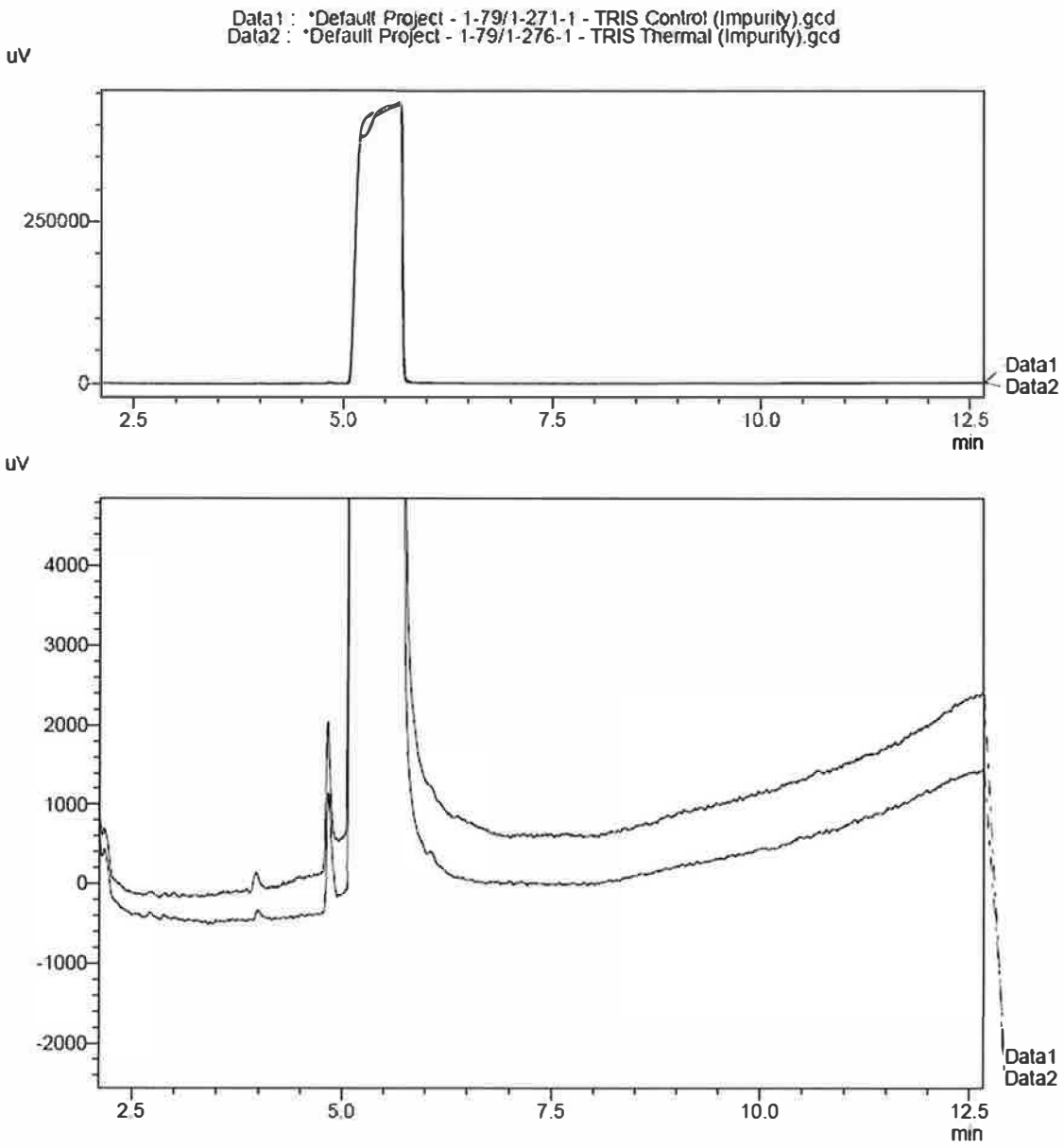


Figure 6: TRIS Thermal Chromatograms

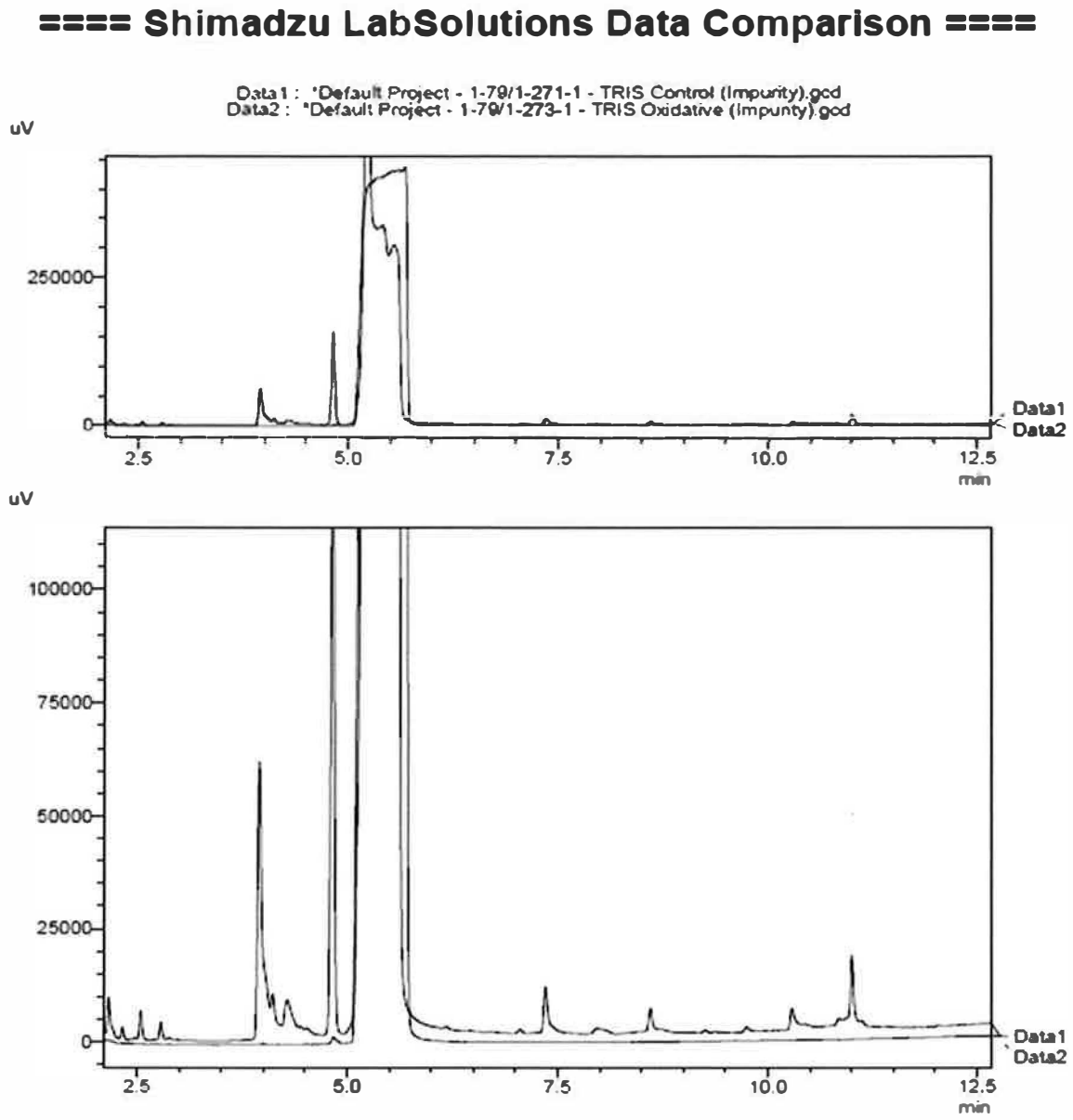


Figure 7: TRIS Oxidative Chromatograms

9. CONCLUSION:

9.1. Tromethamine Degradation product Method Validation

9.1.1. In conclusion, the Tromethamine 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. As an 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.

9.1.2. Assay was only performed in accordance with the protocol to execute the stress study to achieve mass balance. The Assay will be performed via titration for routine and stability analysis and will not be performed via GC-FID outside the scope of this report.

9.2. Deviations from the Validation Protocol

9.2.1. 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.

9.2.2. 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. The recoveries for the low-level samples were 66% for the 0.1% level samples as seen in Section 8.8.4, 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 between all stressed conditions was achieved, for the oxidative sample the 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.