CHAPTER 5

DETERMINATION OF GENOTOXIC IMPURITIES IN ANTI ULCER DRUG: PANTOPRAZOLE SODIUM SESQUIHYDRATE

5.1 INTRODUCTION

Pantoprazole sodium sesquihydrate (Figure 5.1)[165] is an antiulcer and gastric acid inhibitor drug. Pantoprazole sodium sesquihydrate is a medicine used for the treat certain stomach and esophagus.

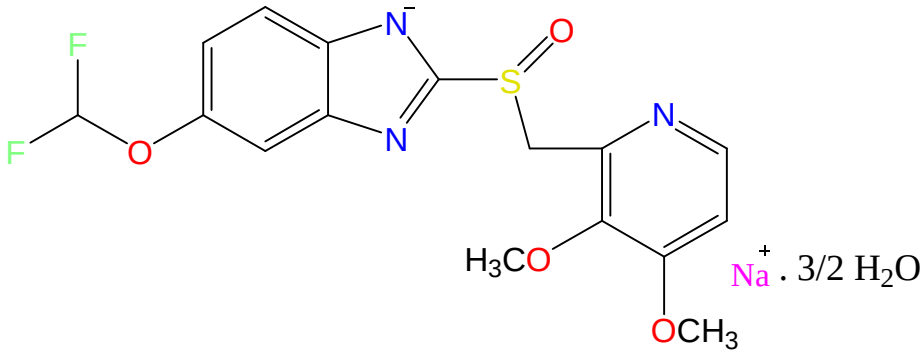

Figure 5.1: The structure of Pantoprazole sodium sesquihydrate

Chemical Name	**:**	5-(difluoromethoxy)-2-[[(3,4-dimethoxy-2-yridyl) methyl] sulfinyl] benzimidazole, sodium salt, sesquihydrate
Molecular formula	**:**	$C_{16}H_{14}F_2N_3NaO_4S.1.5\ H_2O$
Molecular weight	**:**	432.37
CAS No.	**:**	164579-32-2
Melting point	**:**	195 ºC
Phase	**:**	Solid and oral
Appearance	**:**	Off white crystalline powder
Solubility	**:**	Soluble in water acetone, ethyl acetate, isopropanol, chloroform, ethanol and methanol. Insoluble in n-hexane.
Brand names	**:**	Pantoloc, protonix, protonix I.V.

N-(4-hydroxyphenyl) acetamide (GTI-A), N-(4-(difluoromethoxy) phenyl) acetamide (GTI-B) and 4-(difluoromethoxy)-2-nitroaniline (GTI-C) (Figure 5.2) chemicals were used in pantoprazole sodium synthetic process at early stage. These three impurities were potential genotoxic impurities identified from Derek nexus software. Through the regulatory authorities proposed limit to be 6.0 ppm for three impurities in the drug substance based on the drug daily dosage.

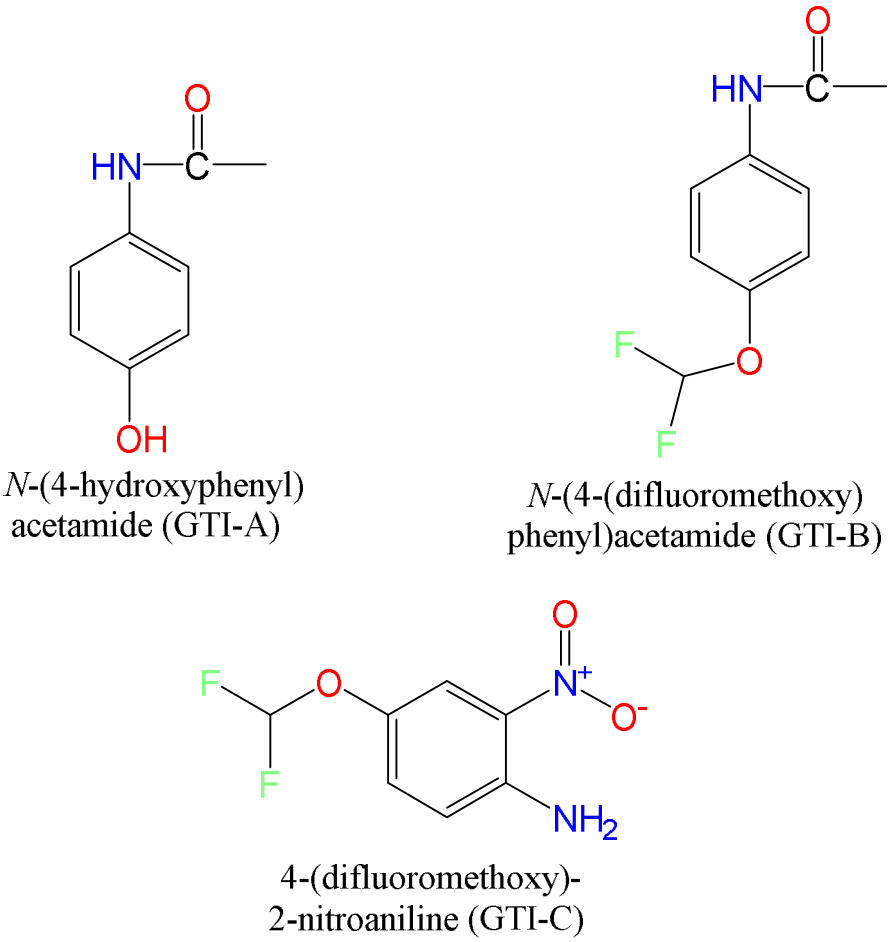

Figure 5.2: The structure of three genotoxic impurities

5.2 EXPERIMENTAL

5.2.1 Standards and chemicals

The following standards and chemicals were used for the genotoxic impurities evaluation, which is mentioned in the table 5.1.

Table 5.1: List of standards and chemicals

S.No.	Chemicals/standards	Grade	Make
1.	Formic acid	LCMS	Merck, Mumbai, India.
2.	Acetonitrile	LCMS	Merck, Mumbai, India.
3.	Water	Mill Q water	Millipore, USA
4.	Pantoprazole sodium sesquihydrate	---	Cipla, Research and development, India.
5.	GTI-A, GTI-B and GTI-C	---	Cipla, Research and development, India.

5.2.2 Preparation of solutions

5.2.2.1 Preparation of standard stock solution

10 mg of each genotoxic test standard taken separately into three different individual 100 mL volumetric flasks and dissolved and diluted up to the mark with diluent and 1 mL of the above solutions transferred into10 mL volumetric flask and diluted upto the mark with diluent. Further diluted 1.0 mL of above solution to 100 mL with diluent and mixed.

5.2.2.2 Preparation of standard solution

6.0 mL of the above standard stock solution is taken into 100 mL volumetric flask and diluted up to the mark with diluent to get the standard solution equivalent to 6 ppm with respect to test concentration of 1 mg/mL.

Calculations for impurity concentrations in ppm

$$\text{Concentration}\ (\text{ppm}) = \frac{\text{Std. weight}}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{6}{100} \times \frac{1}{\text{Spl. conc.}\ (\text{mg/mL})} \times \frac{\text{Purity}}{100} \times 10^6$$

i.e.

$$\text{GTI - A concentration}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{6}{100} \times \frac{1}{1} \times \frac{99.2}{100} \times 10^6$$

$$\text{GTI - B concentration}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{6}{100} \times \frac{1}{1} \times \frac{99.4}{100} \times 10^6$$

$$\text{GTI - C concentration}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{6}{100} \times \frac{1}{1} \times \frac{98.9}{100} \times 10^6$$

5.2.2.3 Preparation of LOD and LOQ solution

The limit of detection solution was prepared by diluting 0.15 mL of standard stock solution into 100 mL with diluent (equivalent to 0.15 ppm). The limit of quantification solution was prepared by diluting the 0.5 mL of standard solution into 100 mL with diluent (equivalent to 0.5 ppm).

5.2.2.4 Preparation of accuracy solutions

Accuracy at LOQ level (0.5 ppm)

Triplicate samples were prepared by 100 mg of pantoprazole sodium into 100 mL volumetric flask and added 0.5 mL of standard stock solution, dissolved and diluted with diluent.

Accuracy at 50% (3 ppm)

Triplicate samples were prepared by 100 mg of pantoprazole sodium into 100 mL volumetric flask and added 3.0 mL of standard stock solution, dissolved and diluted with diluent.

Accuracy at 100% (6 ppm)

Triplicate samples were prepared by 100 mg of pantoprazole sodium into 100 mL volumetric flask and added 6.0 mL of standard stock solution, dissolved and diluted with diluent.

Accuracy at 150% (9 ppm)

Triplicate samples were prepared by 100 mg of pantoprazole sodium into 100 mL volumetric flask and added 9.0 mL of standard stock solution, dissolved and diluted with diluent.

5.2.2.5 Preparation of linearity solutions

LOQ solution (0.5 ppm)

0.5 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

50% Linearity solution (3 ppm)

3.0 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

75% Linearity solution (4.5 ppm)

4.5 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

100% Linearity solution (6 ppm)

6.0 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

125% Linearity solution (7.5 ppm)

7.5 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

150% Linearity solution (9 ppm)

9.0 mL of standard stock solution transferred into 100 mL volumetric flask and diluted up to the mark with diluent.

5.2.2.6 Solution preparation for method precision, intermediate precision and robustness study

100% spiked sample solution used for the method precision, intermediate precision and robustness study. Intermediate precision was performed with different instrument, different lots of solvents and different column in different day.

5.2.2.7 Preparation for stability of analytical solution

100% spiked sample solution and standard solution were prepared by using the above methods and both kept in cooler temperature at 20°C to check the solution stability.

5.2.2.8 Preparation of sample solution

100 mg of pantoprazole sodium sesquihydrate sample transferred into 100 mL volumetric flask, dissolved and diluted up to the mark with diluent

5.2.3 Instrumentation

The list of instrument/equipment was used for the present investigation discussed in chapter 3, table 3.2.

5.3 RESULTS AND DISCUSSION

5.3.1 Method development

Primarily, the trails were performed using HPLC method with different volatile and phosphate buffers and combination with acetonitrile and methanol by gradient and isocratic mode. The attempts were unsuccessful to attain the required sensitivity and recovery for the trace level of genotoxic impurities (6 ppm). Later to get the sensitivity the detection technique was transformed from UV to Mass detector.

Further the trials with LC-MS/MS method were performed with various columns which included C8, C18, C4, amide, amino and phenyl. In addition, various mobile phases such as ammonium formate, formic acid, ammonium acetate, acetic acid with the combination of methanol and acetonitrile have been tested for better optimisation of method. The finally chromatographic separation was achieved and final LC parameters and mass parameters mentioned in the table 5.2 to 5.4.

5.3.2 Operating conditions of LC/MS/MS

Final optimization conditions of LC and MS parameters are presented in table 5.2 to 5.4.

Table 5.2: LC parameters for genotoxic impurities in pantoprazole sodium

LC parameters	
Mode of flow	Gradient
Column	purosphere star RP 18 e (150 mm X 4.6 mm, 3.0 µm)
Solution-A	0.1% formic acid in water
Solution-B	Acetonitrile
Flow	1.0 mL/min
Inj.volume	10 µL
Column oven temperature	25°C
Sampler cooler temperature	20°C
Run time	15 min.
Sample concentration	1.0 mg/mL
Diluent	Acetonitrile :Water (50:50, *v/v*)

Table 5.3: Gradient programme

Time (min)	**Solution-A(%)**	**Solution-B (%)**
0	68	32
6	68	32
9	5	95
12	5	95
13	68	32
15	68	32

Table 5.4: Mass parameters for genotoxic impurities in pantoprazole sodium

Mass parameters			
Parameter	**GTI-A**	**GTI-B**	**GTI-C**
Probe	ESI	ESI	ESI
Polarity	+Ve (Positive)	+Ve (Positive)	+Ve (Positive)
Declustering potential	30 (volts)	60 (volts)	50 (volts)
Collision energy	22 (volts)	32 (volts)	24 (volts)
Collagen exit potential	12 psi	10 psi	15 psi
Ion spray voltage	5500 (volts)	5500 (volts)	5500 (volts)
Source temperature	450ºC	450ºC	450ºC
Entrance potential	7 (volts)	8 (volts)	9 (volts)
Curtain gas	40 psi	40 psi	40 psi
GS1	50 psi	50 psi	50 psi
GS2	50 psi	50 psi	50 psi
Scan Type	MRM	MRM	MRM
MRM Transition	152.1 >110.0	202.1 > 92.0	205.1 > 137.0
Retention time	1.32 minutes	5.14 minutes	8.58 minutes
Retention time of pantoprazole	2.70 minutes		

5.3.3 Validation study

The developed method was fully validated as per ICH guidelines, and checked the parameters as specificity, limit of detection, limit of quantification, precision, linearity, accuracy, robustness study and solution stability.

5.3.3.1 Specificity

The developed method was checked specificity by injecting blank, individual GTI-A, GTI-B and GTI-C impurities and pantoprazole sodium drug substance. No interference peak was observed at the retention time of GTI-A, GTI-B and GTI-C. The retention time of genotoxic impurities GTI-A, Pantoprazole, GTI-B and GTI-C were eluted at retention time of 1.32, 2.70, 5.14 and 8.58 respectively and specificity chromatogram was shown in the figure 5.3.

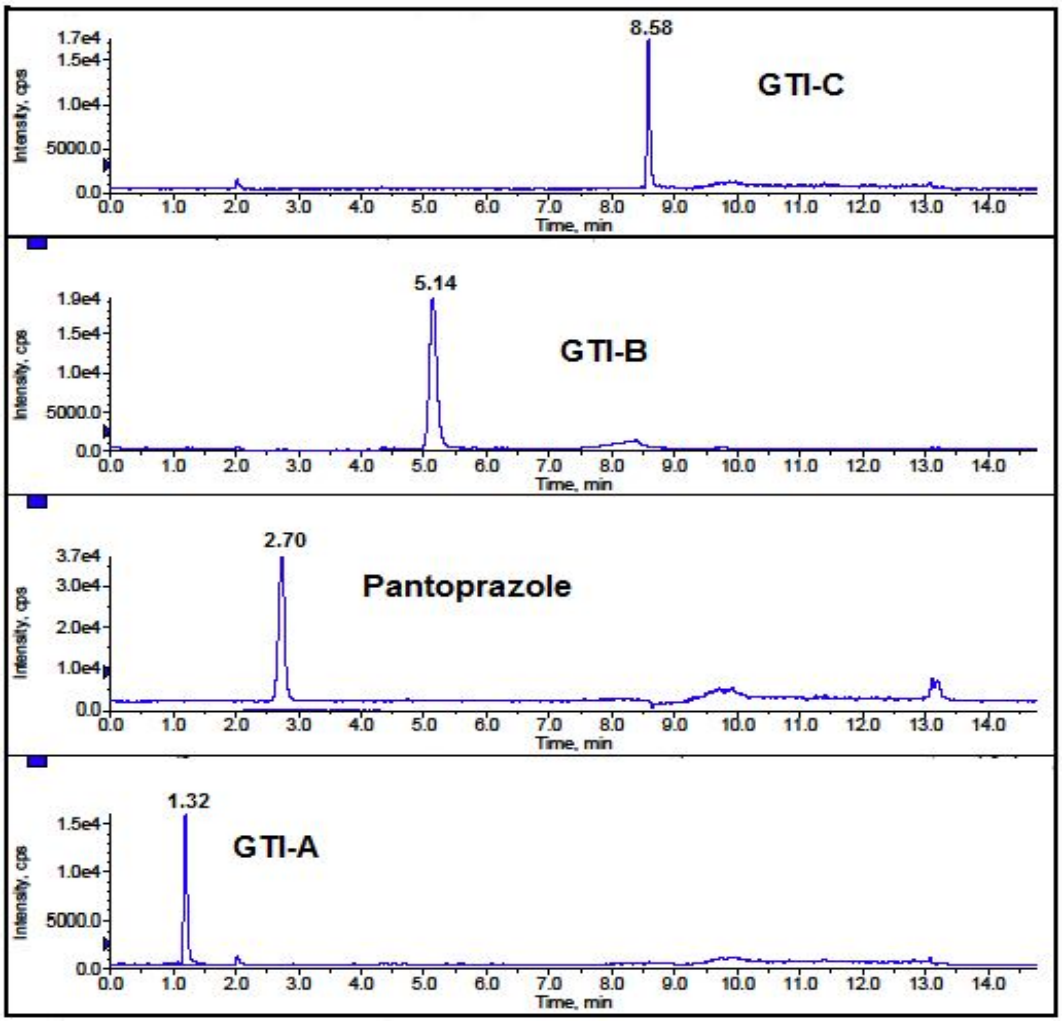

Figure 5.3: Specificity chromatogram

5.3.3.2 Limit of detection (LOD) and limit of quantification(LOQ)

The LOD and LOQ for GTI-A, GTI-B and GTI-C were obtained with the concentration of 0.15 ppm and 0.5 ppm respectively. The corresponding LOD and LOQ values were presented in table 5.5.

Table 5.5: LOD and LOQ data for three genotoxic impurities

Sample name	LOD		LOQ	
	Concentration (ppm)	S/N Ratio	Concentration (ppm)	S/N Ratio
GTI-A	0.15	3.2	0.5	9.9
GTI-B	0.15	3.8	0.5	10.9
GTI-C	0.15	3.6	0.5	10.4

5.3.3.3Linearity

The optimised method was linearity checked with six point calibration graph i.e. LOQ (0.5 ppm), 50% (3.0 ppm), 75% (4.5 ppm), 100% limit level (6.0 ppm), 125% (7.5 ppm) and 150% (9.0 ppm) with respect to 1 mg/mL test concentration. LOQ solution and 150% solution were injected six injections and rest of the levels injected triplicates. The calibration curve was plotted for the concentration (X-axis) versus the peak areas (Y-axis) of analyte. The correlation coefficient, slope and intercept values were found from linear regression analysis. The linearity of the results observed an excellent for all three impurities. The corresponding linearity values and graph represented in table 5.6 and figure 5.4 to 5.6.

Table 5.6: Linearity data for GTI's

Parameter	Result		
	GTI-A	**GTI-B**	**GTI-C**
Linearity range (ppm)	0.5-9	0.5-9	0.5-9
Correlation coefficient	0.9997	0.9999	0.9998
Slope	58941	100899	80641
Intercept	5381	-3218	-4425

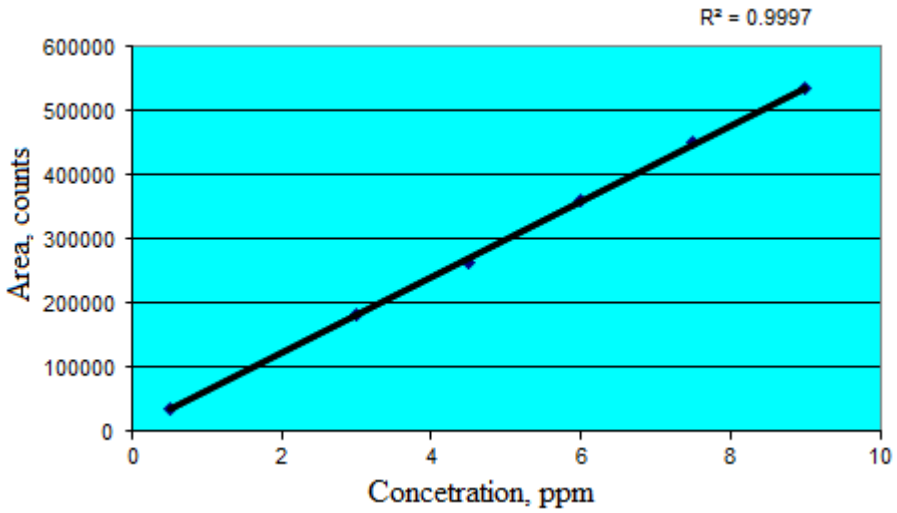

Figure 5.4: Linearity graph for N-(4-hydroxyphenyl) acetamide

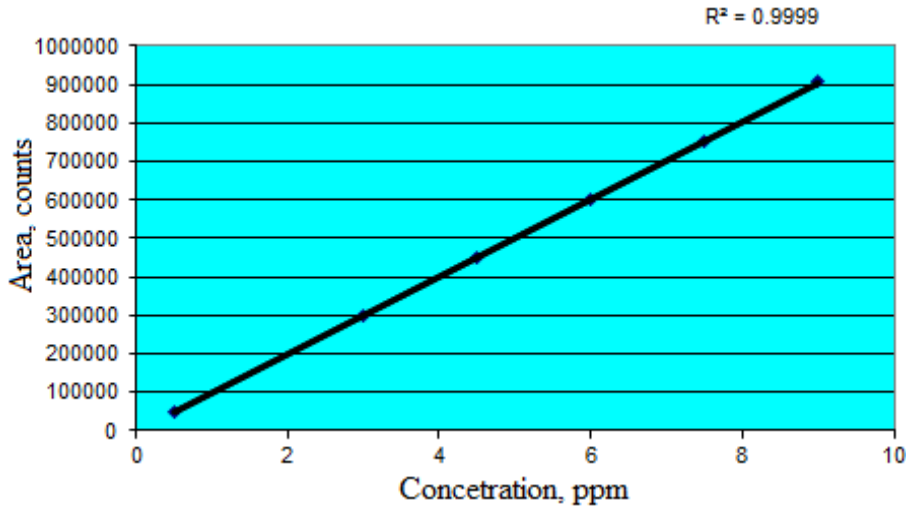

Figure 5.5: Linearity graph for N-(4-(difluoromethoxy) phenyl) acetamide

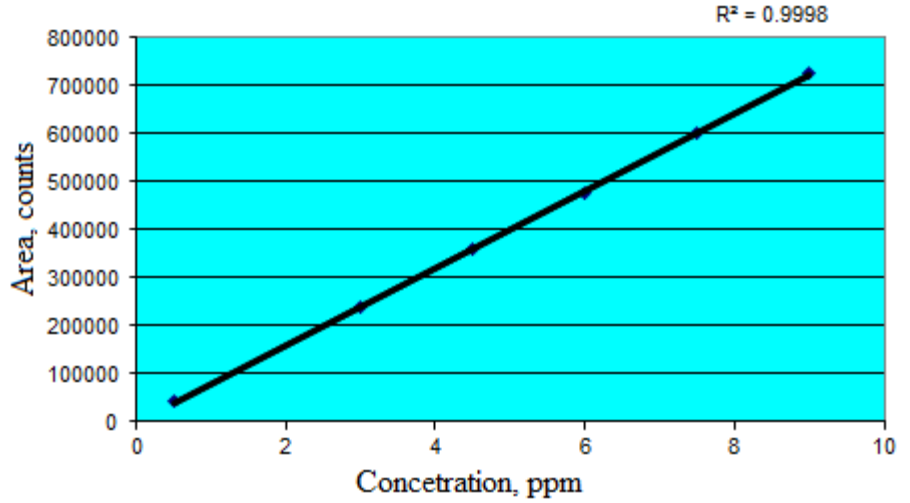

Figure 5.6: Linearity graph for 4-(difluoromethoxy)-2-nitroaniline

5.3.3.4 Accuracy

The accuracy of the method was analysed, triplicate injections were injected at LOQ (0.5 ppm), 50% (3.0 ppm), 100% (6.0 ppm) and 150% (9.0 ppm) level. The three pure sample solutions were injected and impurities were not detected. The recovery values were observed well within the limit (96.3-104.3) for all three genotoxic impurities. The accuracy at such lower level was satisfactory with % RSD > 4.0. The recovery data represented in table 5.7 and corresponding chromatogram at LOQ level was shown in figure 5.7 and 5.8.

Table 5.7: Recovery datafor GTI's

Impurities concentration in ppm	%Recovery of pure samples*		
	Sample-1	**Sample-2**	**Sample-3**
GTI-A			
0.5 ppm	99.3 ± 2.32	97.3 ± 2.67	101.3 ± 1.91
3.0 ppm	97.7 ± 1.74	101.3 ± 0.92	98.7 ± 1.09
6.0 ppm	98.3 ± 2.37	102.3 ± 1.32	100.7 ± 1.02
9.0 ppm	101.1 ± 1.12	96.3 ± 1.72	98.8 ± 1.97
GTI-B			
0.5 ppm	99.9 ± 2.41	101.3 ± 3.12	96.7 ± 1.72
3.0 ppm	101.9 ± 1.22	98.3 ± 1.72	97.7 ± 0.98
6.0 ppm	103.3 ± 1.09	101.1 ± 1.41	98.7 ± 2.12
9.0 ppm	98.8 ± 1.46	102.0 ± 0.92	101.1 ± 1.30
GTI-C			
0.5 ppm	98.3 ± 2.59	99.0 ± 1.62	96.4 ± 2.79
3.0 ppm	96.7 ± 0.62	101.5 ± 1.52	102.3 ± 1.95
6.0 ppm	103.4 ± 0.89	104.3 ± 1.82	100.7 ± 1.39
9.0 ppm	101.9 ± 1.02	96.6 ± 0.59	101.0 ± 1.77

* Mean value of three determinations

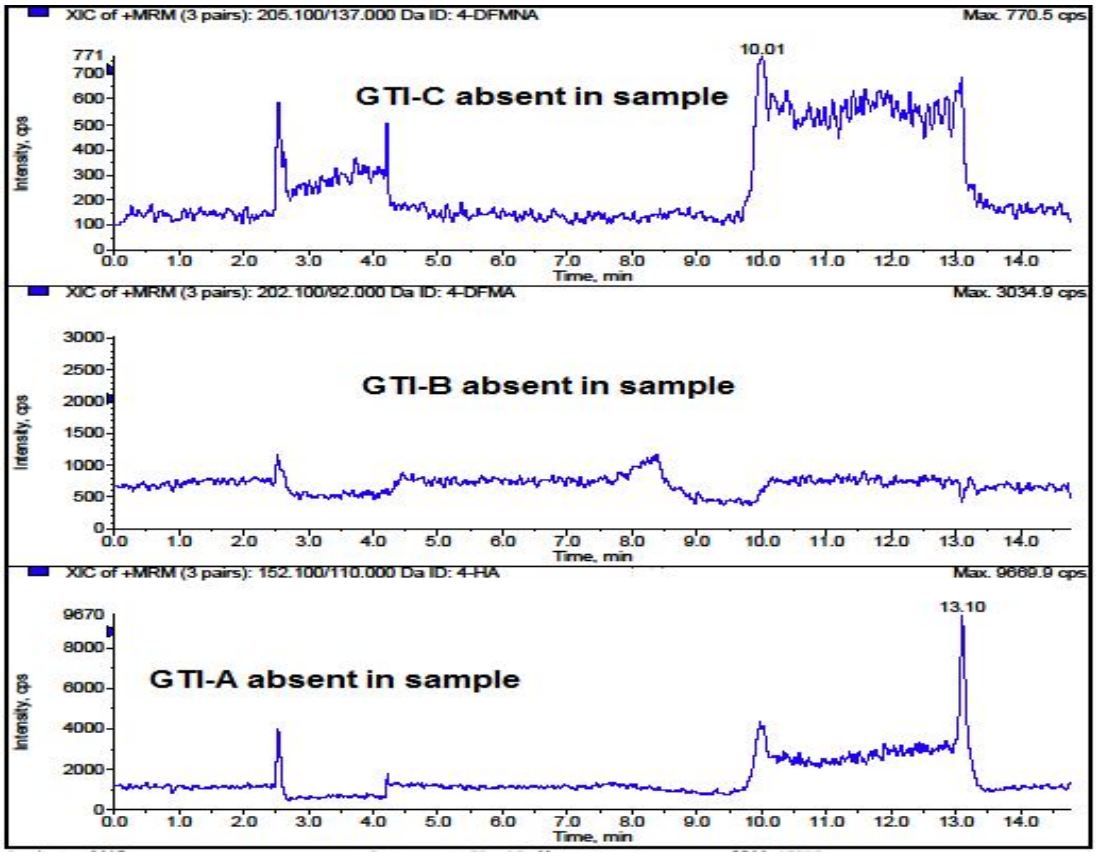

Figure 5.7: Sample chromatogram

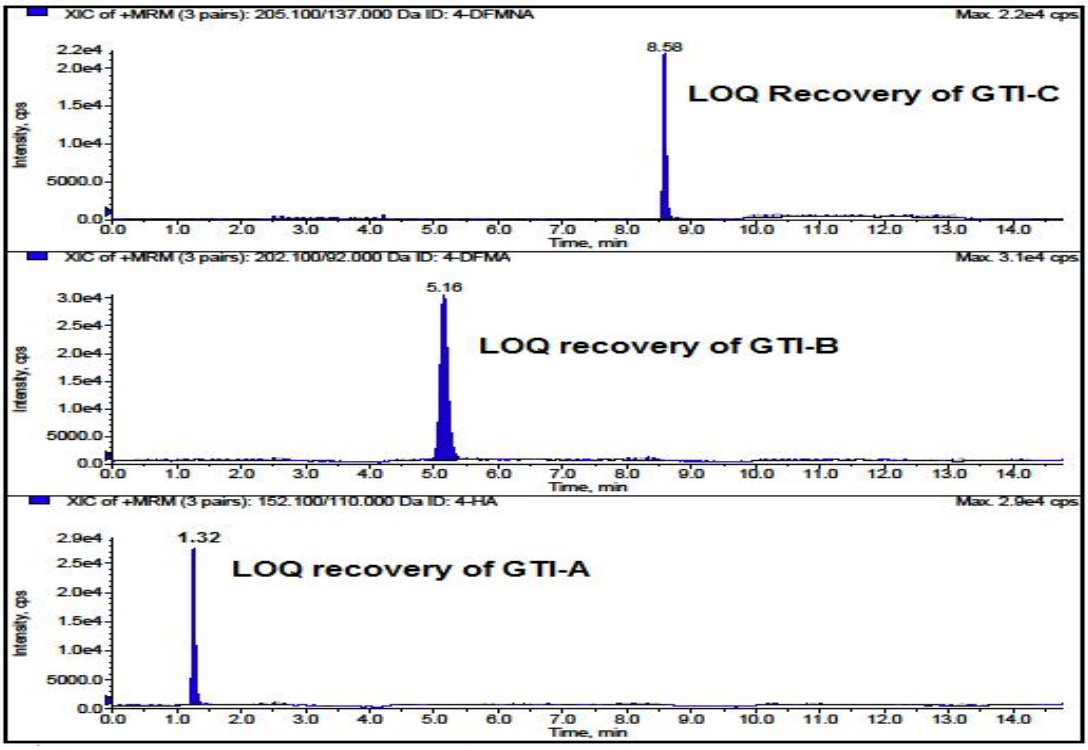

Figure 5.8: Accuracy at LOQ level chromatogram

5.3.3.5 System suitability

The standard solution (mixed three genotoxic impurities) was prepared limit level (6 ppm) with respect to test concentration and injected six times for system precision, before starting the sample analysis. The % RSD value was calculated for areas and observed less than 2.0 % and corresponding results were presented in table 5.8.

Table 5.8: System suitability results

Injection id	GTI-A area	GTI-B Area	GTI-C Area
1	360341	600038	478149
2	351894	607620	475044
3	361007	601432	477122
4	360114	613791	472540
5	360983	601482	474913
6	367848	602288	474976
Avg. area	360365	604442	475457
Std. dev.	5071.7	5279.4	1960.9
%RSD	**1.4**	**0.9**	**0.4**

5.3.3.6 Precision

The precision of the developed LC-MS/MS method for three genotoxic impurities were checked both method precision and intermediate precision. Six different sample solutions were prepared by spiking limit level GTI's with pantoprazole sodium with respect to test concentration 1 mg/mL for method precision and intermediate precision. Intermediate precision was performed with different instrument, different lots of solvents and different column in different day. The %RSD was observed to less than 3.0% for both the impurities in intermediate and method precision, which results confirmed the method is precise.

Table 5.9: Results of precision data

Sample id	GTI-A (ppm)		GTI-B (ppm)		GTI-C (ppm)	
	Method precision	**Intermediate precision**	**Method precision**	**Intermediate precision**	**Method precision**	**Intermediate precision**
1	5.903	5.809	6.192	6.269	5.844	6.144
2	5.998	5.884	6.004	6.136	5.962	6.165
3	5.908	5.801	5.822	6.106	5.990	6.108
4	5.925	5.925	5.925	6.177	5.834	6.198
5	5.967	5.997	5.997	6.146	5.705	6.100
6	5.848	5.677	5.905	6.202	5.988	6.019
Avg.	5.925	5.849	5.974	6.173	5.887	6.122
Std. dev.	0.05	0.11	0.13	0.06	0.11	0.06
%RSD	**0.89**	**1.91**	**2.11**	**0.94**	**1.93**	**1.02**

5.3.3.7 Robustness

Robustness of the method was performed by making small and deliberate changes in operational parameters. The mobile phase flow rate waschanged by 1.1 and 0.9 mL/min(changed by ±0.1 mL/min) and column oven temperature 23°C and 27°C (changed by ±2°C) wereperformed. Test sample spiked with standards at limit level (6.0 ppm) was prepared and injected. The robustness results were presented in table 5.10 to 5.12. The %RSD values were calculated and found to be below 2% for all impurities and do not impact on chromatographic changes, demonstrate that the method was more robust.

Table 5.10: Robustness data for GTI-A

Injection no	GTI-A Peak area				
	Actual condition	Flow		Column temperature	
		Low	High	Low	High
1	360341	364337	353227	356114	367880
2	351894	369982	357339	357009	367997
3	361007	361341	352490	358128	368003
4	360114	360089	354880	357447	368445
5	360983	368842	355114	357401	367331
6	367848	365482	353427	358558	366480
Avg. area	360365	365012	354413	357443	367689
Std. dev.	5071.7	3943.6	1752.0	856.6	691.6
% RSD	**1.4**	**1.1**	**0.5**	**0.2**	**0.2**

Table 5.11: Robustness data for GTI-B

Injection no	GTI-B Peak area				
	Actual condition	Flow		Column temperature	
		Low	High	Low	High
1	600038	605432	582456	612234	592347
2	607620	602438	584391	614438	591402
3	601432	607725	586523	614980	594608
4	613791	608882	584357	615734	594475
5	601482	602856	589823	617390	592240
6	602288	605634	581430	613568	593031
Avg. area	604442	605495	584830	614724	593017
Std. dev.	5279.4	2560.4	3013.6	1778.3	1289.8
% RSD	**0.9**	**0.4**	**0.5**	**0.3**	**0.2**

Table 5.12: Robustness data for GTI-C

Injection no	GTI-C Peak area				
	Actual condition	Flow		Column temperature	
		Low	High	Low	High
1	478149	483455	470124	469125	480229
2	475044	481439	473900	468347	483498
3	477122	484390	479257	465400	481400
4	472540	483466	478322	463421	484432
5	474913	485230	474903	470233	483622
6	474976	486891	475438	467702	482881
Avg. area	475457	484145	475324	467371	482677
Std. dev.	1960.9	1845.9	3277.6	2522.6	1569.2
% RSD	**0.4**	**0.4**	**0.7**	**0.5**	**0.3**

5.3.3.8 Solution stability

Sample solution was prepared as per the proposed method. To this sample, GTI-A, GTI-B and GTI-C were quantitatively spiked at limit level concentration and stored at 20ºC. The standard solution limit level and spiked sample were injected into system immediately and at various intervals. The % recovery of genotoxic impurities in the initial and each interval were calculated by using the following equation and respective results were presented in table 5.13. Good recovery values observed in the range of 96.4-103.2. This indicates that the standard solution and spiked solution were stable up to 34 hours at 20°C.

Calculations for recovery in solution stability

$$\text{Found conc. (ppm)} = \frac{\text{Area observed at various conditions}}{\text{Standard area}} \times \text{Theoretical conc. (ppm)}$$

$$\%\,\text{Recovery} = \frac{\text{Found conc.(ppm)}}{\text{Theoretical conc.(ppm)}} \times 100$$

Table 5.13: The solution stability data for three genotoxic impurity solutions and spiked sample

Conditions	% Recovery		
	GTI-A	**GTI-B**	**GTI-C**
Standard at 0 hrs	99.4	101.2	103.2
Standard at 34 hrs	98.3	99.8	101.5
Spiked sample at 0 hrs	98.4	99.9	97.6
Spiked sample at 34 hrs	97.5	98.3	96.4

5.3.3.9 Application of the Method

If the sample has any detectable GTI-A, GTI-B and GTI-C, then we can calculate in terms of the ppm by using the equation discussed in chapter 3, 3.3.3.9 and three genotoxic impurities were not detected in sample solutions.