CHAPTER 6

EVALUATION OF GENOTOXIC IMPURITIES IN ANTHELMINTIC DRUG: ALBENDAZOLE

6.1 INTRODUCTION

Albendazole (figure 6.1) [166] is an orally administered anthelmintic drug. Albendazole is a medicine used for the treatment for different type of parasitic worm infestations. Albendazole is useful for filariasis, giardiasis, trichuriasis, ascariasis and neurocysticercosis.

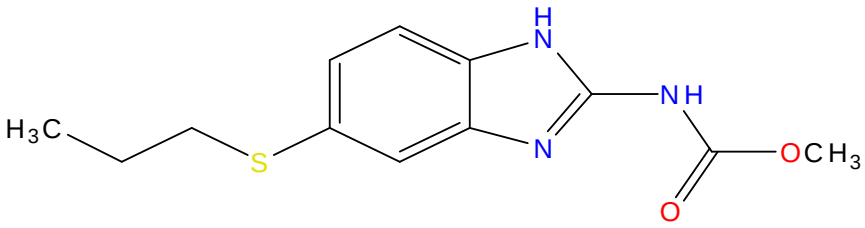
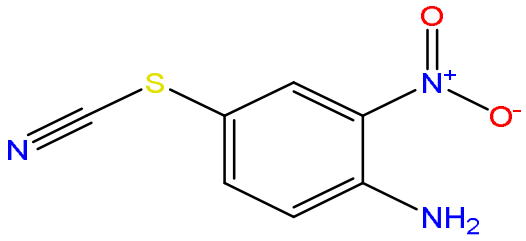


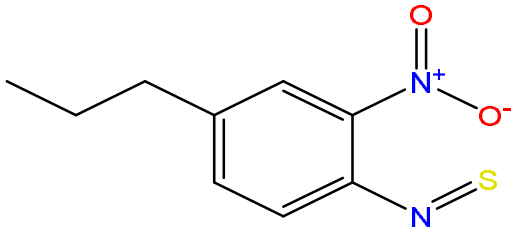
Figure 6.1: The structure of Albendazole

Chemical Name	**:**	Carbamic acid, [5-(propylthio)-1H-benzimidazole-2-yl]-, methyl ester
Molecular formula	**:**	$C_{12}H_{15}N_3O_2S$
Molecular weight	**:**	265.33
CAS No.	**:**	54965-21-8
Melting point	**:**	208 to 210 ºC
Density	**:**	1.3 g/cm^3
Phase	**:**	Solid and Oral
Appearance	**:**	Off white powder
Solubility	**:**	Soluble in dimethylsulfoxide, strong acids and strong bases. Slightly soluble in methanol and chloroform. Insoluble in water.
Brand names	**:**	Albenza,Alworm, Andazol, Eskazole, Noworm, Zentel, Alben-G, ABZ, Cidazole, Wormnil

2-Nitro-4 thio cyanato aniline (GTI-I) and 2-Nitro-4-propyl thio aniline (GTI-II) (figure 6.2) were identified that potential genotoxic impuritiesusing DEREK nexus software.Though GTI-I and GTI-II are known potential carcinogen. The regulatory authorities proposed limit to be 1.8 ppm for GTI-I and GTI-II in the drug substance based on the drug daily dosage.It is necessary to control and prove that these impurities are not carrying forward to till final drug.



2-Nitro-4 thio cyanato aniline (GTI-I)



2-Nitro-4-propyl thio aniline (GTI-II)

Figure 6.2: The structure of GTI-I and GTI-II

6.2 EXPERIMENTAL

6.2.1 Standards and chemicals

The following standards and chemicals were used for the evaluation, which is mentioned in the table 6.1.

Table 6.1: List of standards and chemicals

S.No.	Chemical/standard	Grade	Make
1.	Formic acid	LCMS	Merck, Mumbai, India.
2.	Ammonium formate	LCMS	Merck, Mumbai, India.
3.	Methanol	LCMS	Merck, Mumbai, India.
4.	Acetonitrile	LCMS	Merck, Mumbai, India.
5.	Water	Mill Q water	Millipore, USA
6.	Albendazole	---	Cipla, Research and development, India.
7.	2-Nitro-4 thio cyanato aniline	---	Cipla, Research and development, India.
8.	2-Nitro-4-propyl thio aniline	---	Cipla, Research and development, India.

6.2.2 Preparation of solutions

6.2.2.1 Sample preparation

100 mg of albendazole sample transferred into 10 mL volumetric flask and diluted upto the mark with diluent.

6.2.2.2 Preparation of standard stock solution

10 mg of 2-Nitro-4 thio cyanato aniline and 2-Nitro-4-propyl thio aniline individually transferred into separate 100 mL volumetric flasks, dissolved and diluted upto the mark with diluent. Transferred each 1 mL of the above solution to 10 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.

6.2.2.3 Preparation of standard Solution

1.8 mL of the above standard stock solution transferred into 10 mL volumetric flask and diluted upto the mark with diluent, which is equivalent to 1.8 ppm with respect to test concentration of 10 mg/mL.

Calculations for impurity concentrations in ppm

$$\text{Concentration}\ (\text{ppm}) = \frac{\text{Std. weight}}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{1.8}{10} \times \frac{1}{\text{Spl. conc.}\ (\text{mg/mL})} \times \frac{\text{Purity}}{100} \times 10^6$$

$$\text{GTI - I}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{1.8}{10} \times \frac{1}{10} \times \frac{99.7}{100} \times 10^6$$

$$\text{GTI - II}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{10} \times \frac{1}{100} \times \frac{1.8}{10} \times \frac{1}{10} \times \frac{98.7}{100} \times 10^6$$

6.2.2.4 Preparation of LOD and LOQ solution

Detection limit solution was prepared by diluted 0.15 mL of **s**tandard stock solution into 10 mL with diluent (equivalent to 0.15 ppm). Quantification limit solution was prepared by diluted 0.5 mL of **s**tandard stock solution into 10 mL with diluent (equivalent to 0.5 ppm).

6.2.2.5 Preparation of accuracy solutions

Accuracy at LOQ (0.5 ppm)

Triplicate samples were prepared by 100 mg of albendazole transferred into 10 mL volumetric flask and added 0.5 mL of standard stock solution and diluted upto the mark with diluent.

Accuracy at 50% (0.9 ppm)

Triplicate samples were prepared by 100 mg of albendazole transferred into 10 mL volumetric flask and added 0.9 mL of standard stock solution and diluted upto the mark with diluent.

Accuracy at 100% (1.8 ppm)

Triplicate samples were prepared by 100 mg of albendazole transferred into 10 mL volumetric flask and added 1.8 mL of standard stock solution and diluted upto the mark with diluent.

Accuracy at 200% (3.6 ppm)

Triplicate samples were prepared by 100 mg of albendazole transferred into 10 mL volumetric flask and added 3.6 mL of standard stock solution and diluted upto the mark with diluent.

6.2.2.6 Solution preparation for linearity

LOQ solution (0.5 ppm)

Transferred 0.5 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent (equivalent to 0.5 ppm with respect to test concentration 10 mg/mL).

50% Linearity solution (0.9 ppm)

Transferred 0.9 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

100% Linearity solution (1.8 ppm)

Transferred 1.8 mL of standard stock solution 10 mL volumetric flask, dissolve and diluted upto the mark with diluent.

150% Linearity solution (2.7 ppm)

Transferred 2.7 mL of standard stock solution 10 mL volumetric flask, dissolve and diluted upto the mark with diluent.

200% Linearity solution (3.6 ppm)

Transferred 3.6 mL of standard stock solution 10 mL volumetric flask, dissolve and diluted upto the mark with diluent.

6.2.2.7 Solution preparation for method precision, intermediate precision and robustness

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

6.2.2.8 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 15°C to check the solution stability.

6.2.3 Instrumentation

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

6.3 DISCUSSION ON RESULTS

6.3.1 Method development

There are severalliteratures reports available for the quantification of albendazole in oral suspension and pharmaceutical dosage forms. As per literature review no literature available for the quantification of 2-Nitro-4 thio cyanato aniline and 2-Nitro-4-propyl thio aniline content in Albendazole. The LC-MS/MS technique used for better sensitivity. The finally chromatographic separation was achieved and final chromatographic parameters and mass parameters mentioned in the table 6.2. Before obtaining the final method, the method was analyzed with different columns which includes Phenyl, C18, C8, cyano and amino. In addition to that the trails were passed outthrough various mobile phase additives such as formic acid, acetic acid, ammonium acetate and mixture of methanol and acetonitrile.

6.3.2 LC-MS/MS operating conditions

The final operating condition of the current method LC and mass parameters mentioned in table 6.2.

Table 6.2:LC and mass parameters for genotoxic impurities in albendazole

LC parameters	
Mode of flow	Isocratic
Column	Waters X-Bridge shield RP 18 (25 mm x 4.6 mm ID,3.5µm)
Solution-A	0.01M ammonium formate in water, adjusted pH 6.0 with formic acid solution
Solution-B	Methanol : acetonitrile 50:50 (*v/v*)
Mobile phase	Solution-A : Solution-B, 40:60 (v/v)
Flow	1.0 mL/min
Inj.volume	10 µL
Column oven temperature	40°C
Sampler cooler temperature	5°C

Mass parameters		
Parameter	**GTI-I**	**GTI-II**
Probe	ESI	ESI
Polarity	-Ve (Positive)	-Ve (Positive)
Declustering potential	-40 (volts)	-23 (volts)
Collision energy	20 (volts)	19 (volts)
Collagen exit potential	16 psi	11 psi
Ion spray voltage	-4500 (volts)	-4500 (volts)
Source temperature	450ºC	450 ºC
Entrance potential	-10 (volts)	-12 (volts)
Curtain gas	40 psi	40 psi
GS1 & GS2	50 psi	50 psi
Scan Type	MRM	MRM
MRM Transition	194.0 >163.0	211.0 > 168.0
Sample concentration	10 mg/mL	
Solution-A	2% formic acid in water	
Solution-B	Methanol	
Diluent	Solution A : Solution B (20:80, *v/v*)	

6.3.3 Validation Study

The optimised method was validated in terms of specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, robustness and solution stability as per ICH guidelines.

6.3.3.1 System suitability

The standard solution was prepared limit level (1.8 ppm) with respect to test concentration and six replicate injections were injected for system precision, before starting the sample analysis. The %RSD value was calculated for areas and observed less than 1.0% and corresponding results were presented in table 6.3

Table 6.3: System suitability results

Injection no	GTI-I peak area	GTI-II peak area
1	261340	284355
2	264337	281487
3	267134	283149
4	265142	283266
5	263542	282410
6	264124	283149
Avg. area	264270	282969
Std. dev.	1902.8	957.4
% RSD	**0.7**	**0.3**

6.3.3.2. Specificity

The method was checked by injecting 1.8 ppm solutions of albendazole, GTI-I and GTI-II with respect to test concentration for specificity. The retention time of albendazole, GTI-I and GTI-II observed at retention time of 6.81, 5.29 and 12.22 minutes respectively. The chromatograms for specificity show in figure.6.3 and 6.4. There was no interference at the retention time of GTI-I and GTI-II, which is indicating that the method is specific.

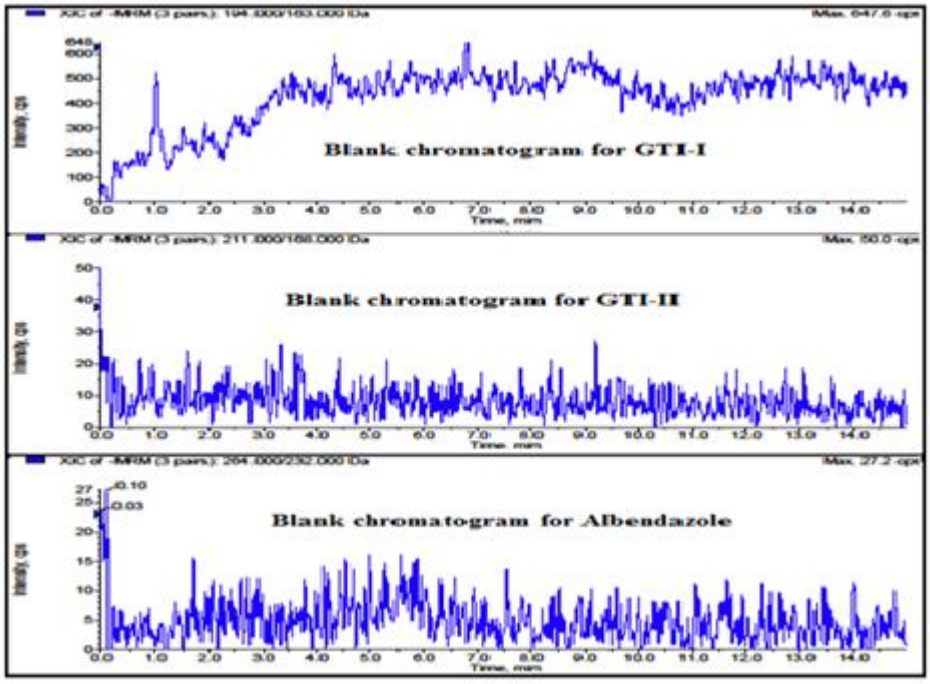


Figure 6.3: Specificity chromatogram for blank

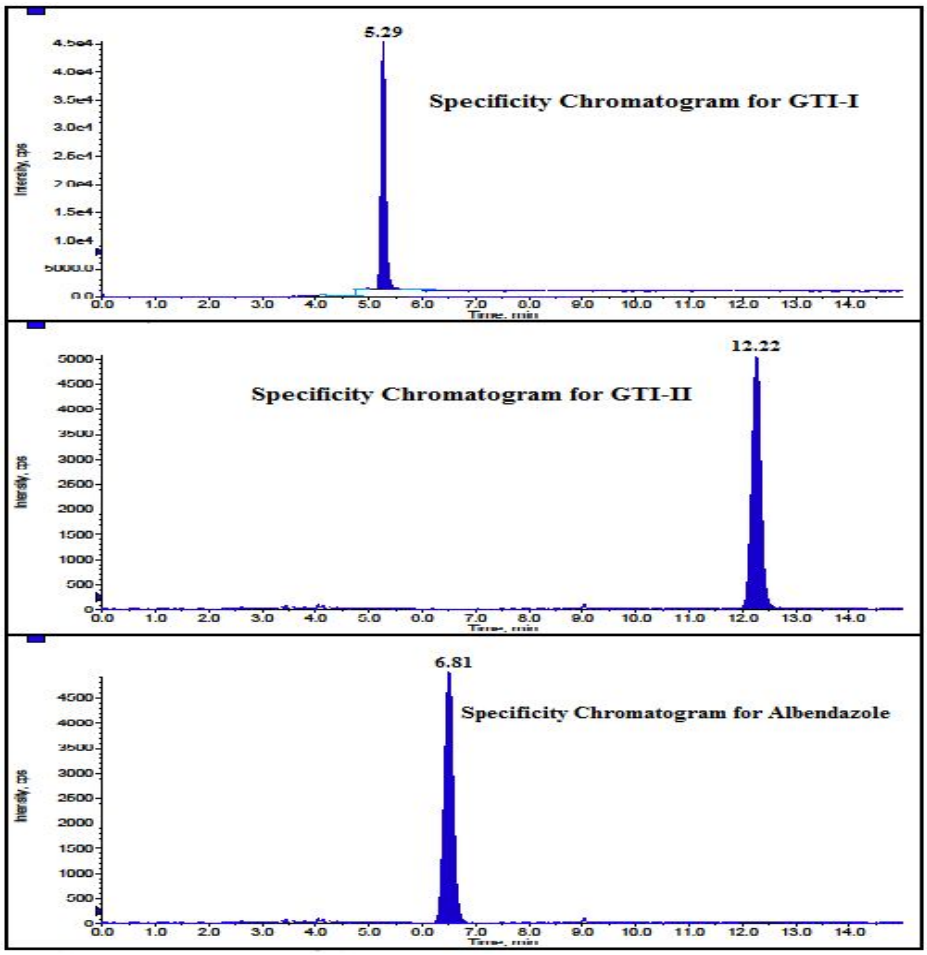


Figure 6.4: Specificity chromatograms for albendazole, GTI-I and GTI-II

6.3.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for GTI-I and GTI-II were obtained with the concentration of 0.15 ppm and 0.5 ppm respectively, which was shown in table 6.4.

Table 6.4: LOD and LOQ data forboth GTI's

Impurity name	LOD Concentration (ppm)	S/N Ratio	LOQ Concentration (ppm)	S/N Ratio
GTI-I	0.15	3.3	1.0	9.8
GTI-II	0.15	3.7	1.0	10.9

6.3.3.4 Linearity

The developed method was confirmed over a concentration of five levels 0.5-3.6 ppm (LOQ, 50%, 100%, 150% and 200%) for linearity. The concentration of ppm in X-axis and peak areas in Y-axis checked for calibration curve. Six replicate injections were injected for LOQ and 200% and triplicate injections were injected for rest of the levels. The correlation coefficient, slope and intercept values were found through regression analysis. Correlation coefficient, slope and intercept values for GTI-I, 0.9999, 78158 and -1893 and for GTI-II, 0.9997, 59974, 3178 and linearity graph is shown in figure 6.5 and 6.6.

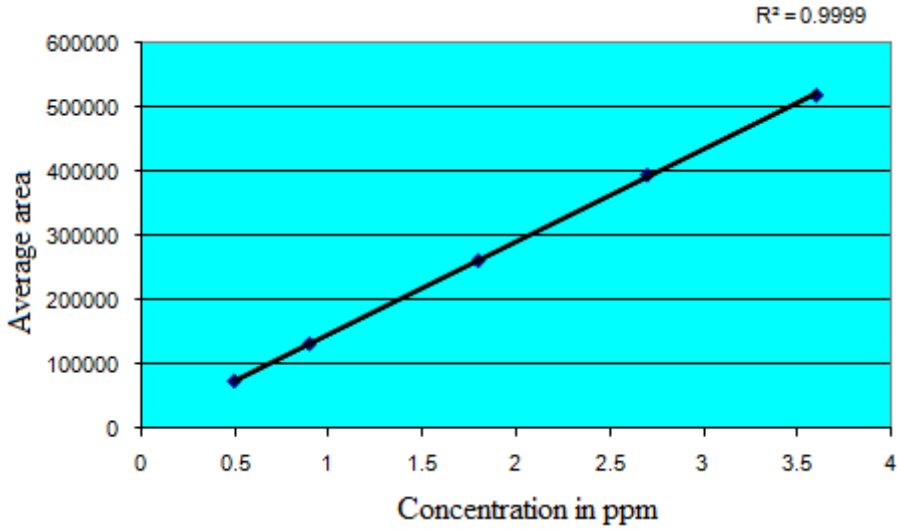


Figure 6.5: GTI-I linearity graph.

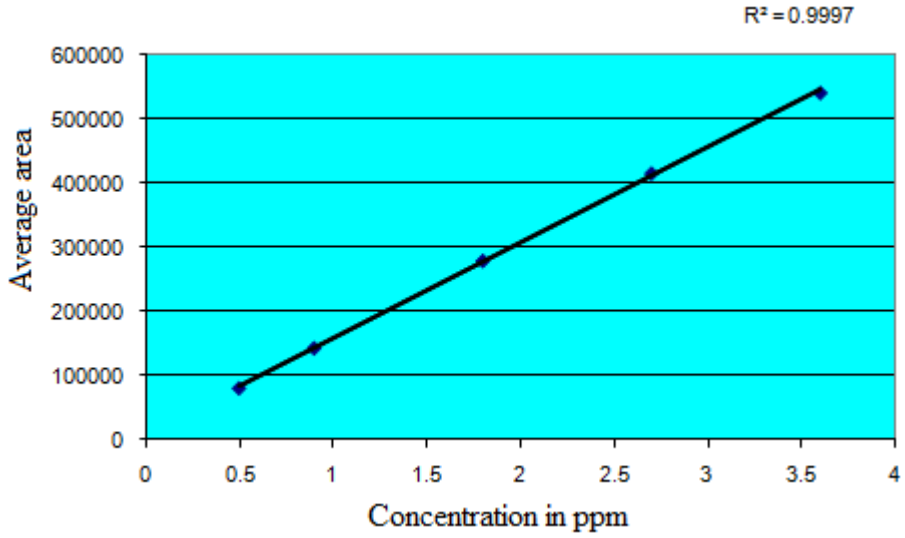


Figure 6.6:GTI-IIlinearity graph.

6.3.3.5 Accuracy

The accuracy of the method was determined triplicate injections at LOQ, 50%, 100% and 150% level. The recovery obtained for both the genotoxic impurities well within the limit and corresponding recovery values were represented in table 6.5. Accuracy chromatogram was shown in figure. 6.7.

Table 6.5: Accuracy data for GTI-I and GTI-II

% Spiked	**GTI-I**		**GTI-II**	
	% Recovery	**% RSD**	**% Recovery**	**% RSD**
LOQ level	96.4	**2.99**	99.9	**3.41**
	102.3		103.9	
	100.1		97.1	
50% level	101.1	**2.37**	97.2	**3.26**
	98.7		99.9	
	103.5		103.7	
100% level	97.1	**3.42**	102.9	**3.17**
	103.9		96.7	
	101.5		98.8	
200% level	103.8	**2.02**	99.5	**2.84**
	102.1		103.1	
	99.7		97.5	

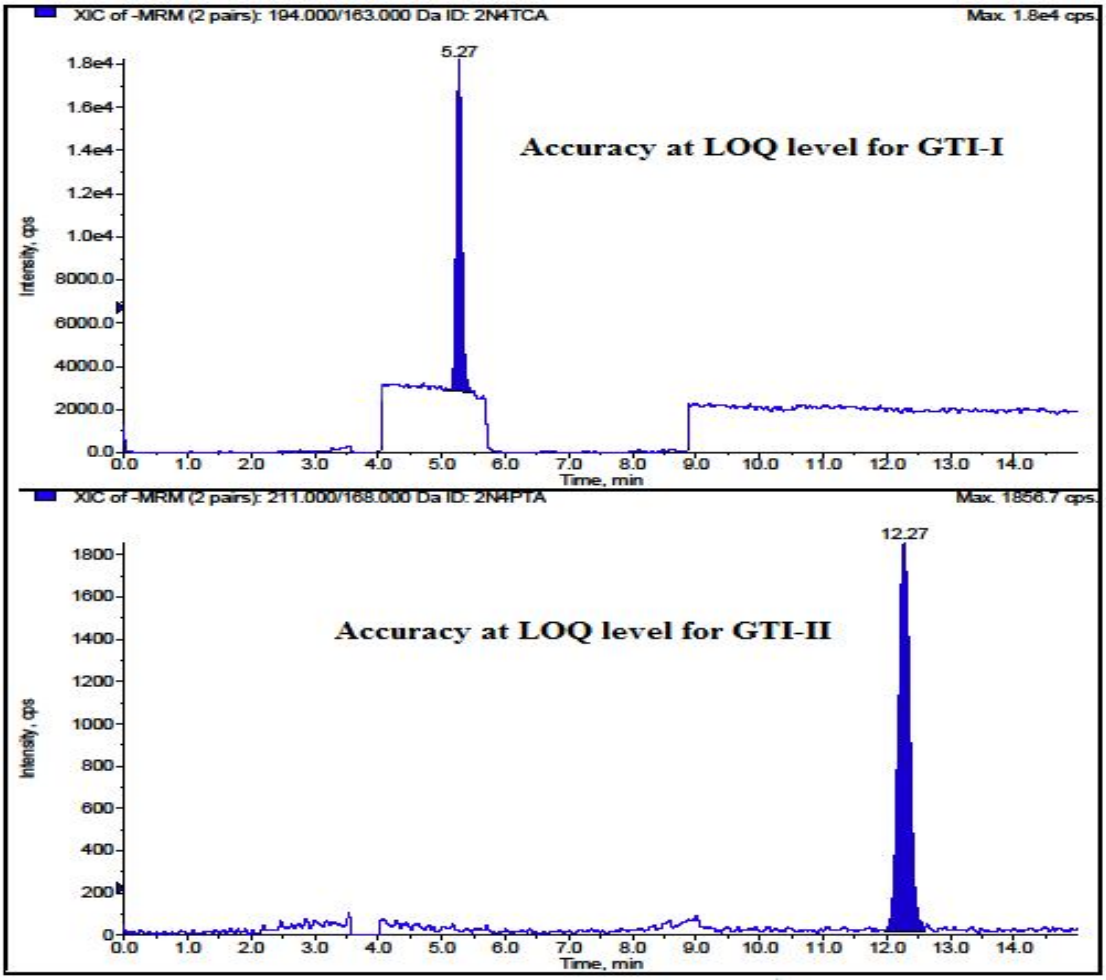


Figure 6.7: Accuracy chromatogram for GTI-I and GTI-II

6.3.3.6 Precision

Six individual solutions were prepared by spiking with albendazole limit level GTI impurities with respect to albendazole 10 mg/mL concentration 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 2.0% for both the impurities in method precision and intermediate precision, which results confirmed the method is precise.

Table 6.6: Method precision and intermediate precision for GTI-I and GTI-II

Sample id	Method precision		Intermediate precision	
	GTI-I (ppm)	**GTI-II (ppm)**	**GTI-I (ppm)**	**GTI-II (ppm)**
1	1.854	1.764	1.773	1.818
2	1.883	1.823	1.779	1.743
3	1.834	1.784	1.785	1.801
4	1.853	1.793	1.806	1.822
5	1.834	1.856	1.775	1.809
6	1.849	1.841	1.759	1.722
Avg.	1.851	1.810	1.780	1.786
Std. dev.	0.018	0.036	0.016	0.042
% RSD	**0.97**	**1.97**	**0.88**	**2.38**

6.3.3.7 Robustness

The robustness of the method was determined 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 38°C and 42°C (changed by ±2°C) wereperformed. Results for robustness at various conditions (flow rate and temperature) including %RSD represented in table 6.7 and 6.8, which indicated that the chromatographic performance was not affected by these changes. The %RSD was found to be less than 2.0%.

Table 6.7: Robustness data for various flow rates

	Actual flow		Low flow		High flow	
Injection no	**GTI-I peak area**	**GTI-II peak area**	**GTI-I peak area**	**GTI-II peak area**	**GTI-I peak area**	**GTI-II peak area**
1	261340	284355	268242	276227	253430	290116
2	264337	281487	268005	274332	254005	289014
3	267134	283149	268148	273149	254228	290337
4	265142	283266	268335	275004	253014	289158
5	263542	282410	269117	274118	254337	288298
6	264124	283149	270128	273309	252668	290545
Avg. area	264270	282969	268663	274357	253614	289578
Std. dev.	1902.8	957.4	816.7	1142.9	684.3	887.0
% RSD	**0.7**	**0.3**	**0.3**	**0.4**	**0.3**	**0.3**

Table 6.8: Robustness data for various column temperatures

	Actual temperature		Low temperature		High temperature	
Injection no	**GTI-I peak area**	**GTI-II peak area**	**GTI-I peak area**	**GTI-II peak area**	**GTI-I peak area**	**GTI-II peak area**
1	261340	284355	269143	292134	258339	277497
2	264337	281487	268005	293988	258012	278134
3	267134	283149	271232	294004	259234	279083
4	265142	283266	270143	293056	252375	277005
5	263542	282410	273008	293982	253189	278332
6	264124	283149	271337	294014	251043	279018
Avg. area	264270	282969	270478	293530	255365	278178
Std. dev.	1902.8	957.4	1773.0	780.6	3554.6	823.4
% RSD	**0.7**	**0.3**	**0.7**	**0.3**	**1.4**	**0.3**

6.3.3.8 Solution Stability

GTI-I and GTI-II were quantitatively spiked at limit level concentration of 1.8 ppm and standard solution limit level stored at 5ºC for solution stability. The spiked solution and standard solution at limit level were injected, initially and different intervals. The % recoveries were calculated as per calculations given below and corresponding results represented in table 6.9. No significant change observed for sample and standard solutions, this indicates that the sample solution and standard solutions were stable upto 14 hours at 5ºC.

Calculations for recovery in solution stability studies

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

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

Table 6.9: The solution stability data for both GTI's and spiked sample at different time intervals.

Conditions	**GTI-I Found conc. (ppm)**	**GTI-II (% Recovery)**	**GTI-II Found conc.(ppm)**	**GTI-II (%Recovery)**
Standard initial	1.786	98.5	1.822	100.4
Spiked initial	1.814	100.0	1.791	98.7
Standard 14 hours	1.763	97.2	1.801	99.3
Spiked 14 hours	1.802	99.3	1.768	97.5

6.3.3.9 Method Application

If the sample has any detectable GTI-I and GTI-II impurities, then we can calculate in terms of ppm by using the equation discussed in chapter 3, 3.3.3.9 and both impurities were not detected in sample solution.