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Detection and Quantification of Two Potential Genotoxic Impurities in Telbivudine Active Pharmaceutical Ingredient by Targeted Ultra Performance LC-MS/MS Analysis

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A new selective, rapid and highly sensitive method using reversed-phase ultra-performance liquid chromatography (RP-UPLC) coupled with mass analyzer was developed for the detection and quantification of two genotoxic impurities *viz*. 4-dimethyl aminopyridine (GTI-1) and 2-bromo-3,5-diacetyl thymidine (GTI-2) in Telbivudine drug substance. The separation of two genotoxic impurities was accomplished using Kromasil C8 column (100 mm × 4.6 mm, 3.5 µm) maintained at 45 ºC using formic acid (0.1%) as buffer solution and gradient grade acetonitrile with 0.5 mL/min flow rate. Both GTIs were quantified using positive electrospray ionization (ESI) mode on MS/MS Analyser. ICH guidelines were followed for validation of genotoxic impurities. % RSD for system precision was found to be 2.71 and 2.94 and the overall %RSD for ruggedness was 3.11 and 2.89, respectively for GTI-1 and GTI-2. Two GTIs and Telbivudine drug were well resolved at retention times: 1.76 min for GTI-1, 3.81 min for Telbivudine and 6.62 min for GTI-2. The linearity relationship holds good in the range of concentrations 0.5 ppm to 7.5 ppm for both the impurities with a correlation coefficient of 0.999. Accuracy ranged from 95.1% to 105.1%. The GTI-1 and GTI-2 were shown, respectively detection limits: 0.07 ppm and 0.09 ppm and quantification limits: 0.22 ppm and 0.27 ppm and the solutions were stable upto 24 h at room temperature. The proposed and validated method is sensitive, reproducible, specific and linear and can be successfully applied for the quantification of both genotoxic impurities in Telbivudine active pharmaceutical ingredient and formulation.

Keywords: Telbivudine, Method development, Quantification, Genotoxic impurities, Method validation.

INTRODUCTION

Telbivudine is a synthetic antiviral drug used in the treatment of hepatitis B virus (HBV) infection and more efficient than adefovir or lamivudine drug [1-4]. The key cause of hepatitis worldwide is due to hepatitis B virus [5,6]. The longterm hepatitis B infection is caused in the liver by hepatitis B virus. The continuous presence of infection can cause liver failure, liver damage and rarely liver cancer. Telbivudine drug helps to reduce the amount of hepatitis B virus load in human body [7-14]. Telbivudine, a synthetic nucleoside analog, acts on the hepatitis B virus and inhibits second-strand DNA. The HBV DNA poly-merase (reverse transcriptase) is inhibited by telbivudine 5′-triphosphate, which is prone to the chain termination and this in turn helps to inhibit the replication of the virus [15]. During the process development for manufacturing of active pharma-ceutical ingredients (APIs), the final drug substances may end up inadvertently with some toxic impurities [16]. The genesis of these impurities may be attributed to trace remains of various chemicals employed, intermediates, byproducts of chemical reactions and various other startup processes. These impurities have no therapeutic importance but are potentially harmful. Some of these impurities or chemicals have the potential to damage the genetic information by inducing mutation in the cell, which leads to cancer in humans and are called genotoxic impurities [17,18]. To minimize these, researchers have to identify and quantify the genotoxic potential impurities in the initial stages of process and thereby to have the control on the synthetic process of drug [19]. Of the various analytical procedures, mass spectrometric methods are more sensitive and effective in the quantification of impurities in trace levels. Henceforth, the impurity profiling is powerful and challenge

to the analytical development scientist. For the above reasons, US FDA and EMEA published guidelines separately related to potential genotoxic impurities limits within commercial new drug substances [20,21]. These two agencies have set a threshold of toxicological concern (TTC) of 1.5 µg/day, for potential genotoxic impurities. The key initial compounds used in the product synthesis of Telbivudine are GTI-1 and GTI-2, already identified as genotoxic impurities. The structure of Telbivudine as well as GTIs is shown in Fig. 1. On the basis of threshold, the toxicological concern-limit of 1.5 µg/person/day as well as the highest daily dose of adult of Telbivudine, GTIs are to be controlled at 5 ppm concentration limit in active pharmaceutical substances.

Therefore, there is a necessity to develop sensitive, suitable analytical methods to detect and quantify the necessary limit of 1.5 µg/day consumption of individual GTIs. In the initial stage of pharmaceutical analysis, the conventional methods based on 'HPLC with PDA/UV detection' for non-volatile analytes and 'gas chromatograph with FID detector' for volatile analytes were employed. But in view of complexity of sample matrices and analytes properties and for accurate quantification of analyte at very low concentrations, these methods are often inadequate. In recent days, spectroscopy and mass analytical techniques, have gained popularity because of better sensitivity and mass selective detection. When compared to nonspecific detectors such as the PDA/UV, developing a method using mass analyzer generally are less likely to suffer from the drug interventions due to specificity of detection and thereby, reducing the runtime. According to the literature, few analytical methods have been described for the estimation of Telbivudine and its impurities [22,23], but no analytical approach has been reported for the quantitative estimation of the two discovered GTIs in Telbivudine till date.

Hence, the endeavour of the present investigation is to develop a simple, highly sensitive and more specific method based on UPLC-MS/MS for the quantification of GTI-1 and GTI-2 in Telbivudine drug. Thus developed method is aimed to be validated with respect to precision, ruggedness, recovery, linearity,specificity, detection limit and quantification limits as per the regulatory guidelines of ICH [24,25].

EXPERIMENTAL

Reference substances of Telbivudine, GTI-1 and GTI-2 were assisted from Performics Laboratories Ltd., Hyderabad, India. HPLC grade solvents were procured from Merck (formic acid and acetonitrile). Water from Milli-Q plus system from Millipore was used.

The instrumentation used was Acquity UPLC with an autosampler (Waters H-Class) and PDA detector coupled with Mass Analyzer from Waters, USA (XEVO TQ-S) for developing and validating the method. Data processing and acquisition was carried using MassLynx software.

Standard and sample solution preparation

Formic acid buffer 0.1%: Formic acid (1.0 mL) was added in 800 mL of water, mixed and diluted to 1000 mL and filtered through finer porosity filtered membrane of 0.22 µm.

Preparation of mobile phase: Formic acid buffer (0.1%) was used as "mobile phase-A" and acetonitrile (HPLC grade) as "mobile phase-B".

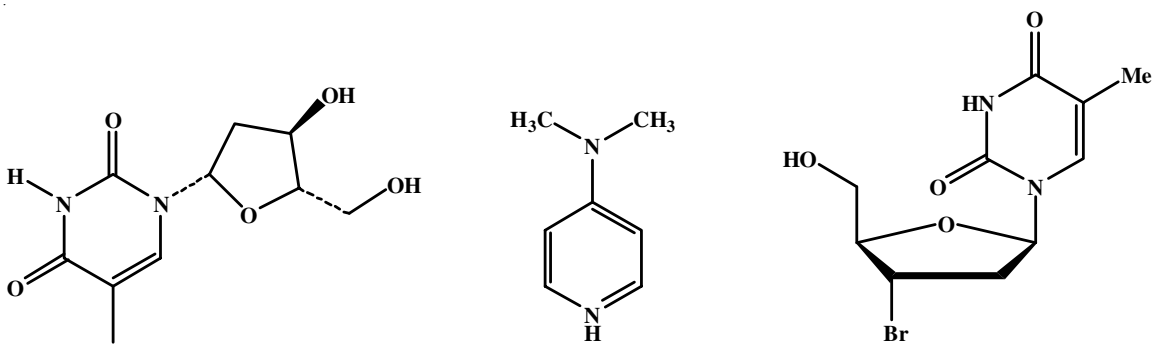
Preparation of diluent: Mixture of water:acetonitrile: 0.1% formic acid buffer (750:250:1.0) v/v/v was used as diluent.

Impurity standards and sample: Two GTI standards solutions (4-dimethyl amino pyridine and 2-bromo-3,5-diacetyl thymidine compound) were prepared at 0.005 ppm in the diluent. Sensitivity solution was prepared at 0.001 ppm. Weighed and transferred an accurate amount of Telbivudine to get a sample concentration of 2000 ppm in diluent.

Chromatographic conditions

LC parameters: The instrumentation used was Acquity UPLC with an autosampler (Waters H-Class) and PDA detector coupled with Mass Analyser from Waters, USA (XEVO TQ-S). The Kromasil C8 (3.5 µm,100 × 4.6 mm,) analytical column was used. The gradient mode of elution was employed with a buffer solution as mobile phase-A and acetonitrile as mobile phase-B at 0.5 mL/min flow rate and oven temperature of column was kept at 45 ºC. A 10 µL of injection volume was used. The gradient was programmed as: Time (min)/% mobile phase A: 0/90, 3/90, 6/25, 11/25, 12/90, 15/90, with an equilibrium time of 2 min. A switching valve program was employed between 3.0 min to 6.2 min to divert the Telbivudine peak to vent.

Mass/mass parameters: Tandem quadrupole mass analyzer (Xevo TQ-S) was equipped with electrospray ionization (ESI) in positive mode with MRM. The data collection and software control was done by MassLynx software. The equipment was set with nebulizer gas 6.0 bar, desolvation gas



Telbivudine **GTI-1** **GTI-2**

Fig. 1. Chemical structure of Telbivudine, GTI-1 and GTI-2

at 800 L/h, cone gas at 150 L/h, desolvation temperature at 500 ºC, cone at 25 Volts, capillary: 3.5 kV and source temperature: 150 ºC, Dwell time was 200 msec and scan range 50-1000 *m/z*. For the quantification of 4-dimethyl amino pyridine:parent ion: 123.11 *m/z* and daughter ion: 107.31 *m/z* and for 2-bromo-3,5-diacetyl thymidine, parent ion 405.11 *m/z*, daughter ion 127.09 *m/z* were used with multiple reaction monitoring (MRM) mode.

RESULTS AND DISCUSSION

Optimization of chromatographic parameters: The aim of the current work is to develop sensitive and specific symmetrical peaks with short runtimes for genotoxic impurities: GTI-1 and GTI-2, using UPLC-MS/MS method. Several attempts were made with various buffers such as ammonium acetate and formic acid with different propositions of methanol and acetonitrile as mobile phases both in isocratic and gradient mode compositions using different reversed-phase stationary phases (C8 and C18) and with various percent of loading in columns as a preliminary run. The separation of Telbivudine and GTIs is critical due to its polarities and structures. At this point, one of the distinct parameters is the selection of diluent to verify the solubility of GTIs and drug substance. The selection of diluent influences recovery, sensitivity and precision. Asymmetric peaks observed with Inertsil-ODS-3V, 5 µm (150 mm × 4.6 mm) and inadequate response with Zorbax SBC18, 5 µm (150 mm × 4.6 mm) with gradient mode of elution found unsatisfactory results. The separation and response of GTIs from Telbivudine were found satisfactory with the Kromasil C8 5 µm (150 mm × 4.6 mm) and impurities were well retained. An excellent peak shape, accurate recovery and good response were observed with a proposition of water: acetonitrile: 0.1% formic acid buffer: 750:250:1.0 v/v/v', as diluent. Two GTIs of Telbivudine were separated on RP UPLC column Kromasil C8 (150 mm × 4.6 mm, 3.5 µm) column with mobile phase-A as formic acid buffer (0.1%) and mobile phase-B as acetonitrile with gradient mode of elution, were found more efficient to achieve well separation and desired peak shape.

Optimization of mass spectrometric parameters: For the analysis of the qualitative and quantitative determination of each impurity chemical structure and mass fragmentation law were used. MRM mode is mostly preferred as its shows a very low limit of detection and eliminates background noise, as it has significant selectivity and sensitivity. The transition at 123.14 *m/z* (parent mass), 107.09 *m/z* (fragment mass) for 4-dimethyl amino pyridine and 2-bromo-3,5-diacetyl thymidine compound at 405.11 *m/z* (parent mass), 127.09 *m/z* (fragment mass) MRM were selected based on response (Fig. 2a-c).

Method validation study parameters: The developed UPLC-MS/MS method was validated for precision, specificity, ruggedness, linearity, accuracy, detection limit, quantification limit and solution stability.

System suitability: System suitability is an integral part of method of analysis. The sensitivity solution and standard solutions were prepared and analyzed as per the optimized method. The signal to noise ratio for the sensitivity solution were in the range of 36.25-57.52 (S/N not less than 15.0) and the %RSD of peaks areas of GTI-1 and GTI-2 from six replicate injections of standard solutions were in the range of 0.97-2.94 and the values were well within the acceptable limit (not more than 15.0%). System suitability results are listed in Tables 1 & 2 and the corresponding representative chromatograms are shown in Figs. 3 and 4.

TABLE-1
SYSTEM SUITABILITY RESULTS

Parameters	GTI-1		GTI-2	
	S/N ratio	%RSD	S/N ratio	%RSD
System precision, MP	46.37	2.71	38.41	2.94
Linearity	52.14	1.07	57.52	0.98
Ruggedness	36.48	0.98	42.95	1.24
Recovery, specificity	53.09	1.26	55.41	1.17
Solution stability initial	48.65	0.97	36.25	2.32
Solution stability after 24 h	39.15	1.14	42.52	1.89
Precision at LOQ	44.56	2.24	38.99	0.97

TABLE-2
SYSTEM PRECISION

Injection No.	Peak area of GTI-1	Peak area of GTI-2
Injection-1	210254	249245
Injection-2	225014	239856
Injection-3	218345	239994
Injection-4	224158	228362
Injection-5	226482	245375
Injection-6	219964	239426
Average	220702.8333	240376.3
%RSD	2.71	2.94

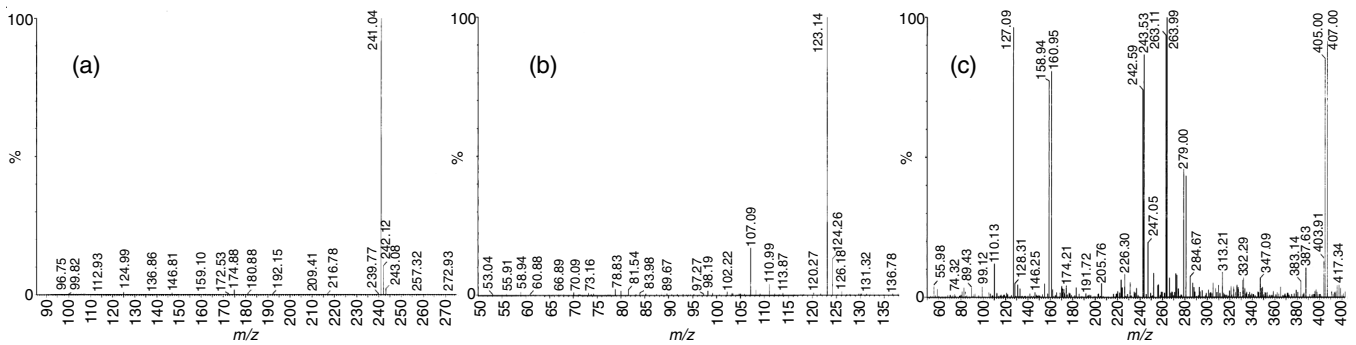


Fig. 2. Mass fragmentation pattern of (a) Telbivudine, (b) GTI-1 and (c) GTI-2

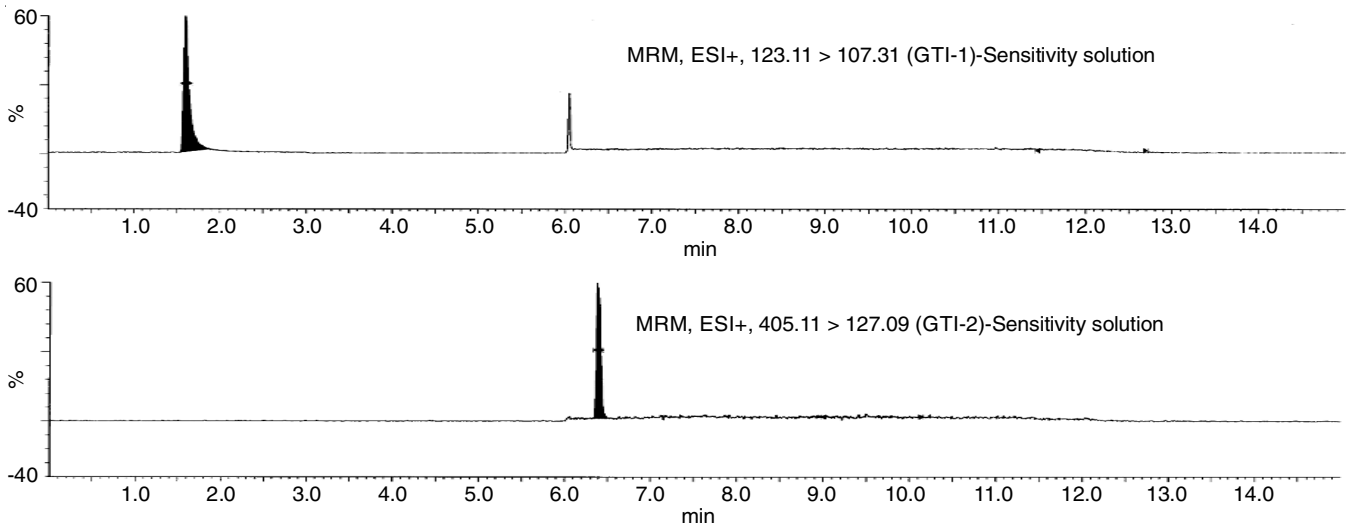


Fig. 3. Sensitivity solution of GTI-1 and GTI-2

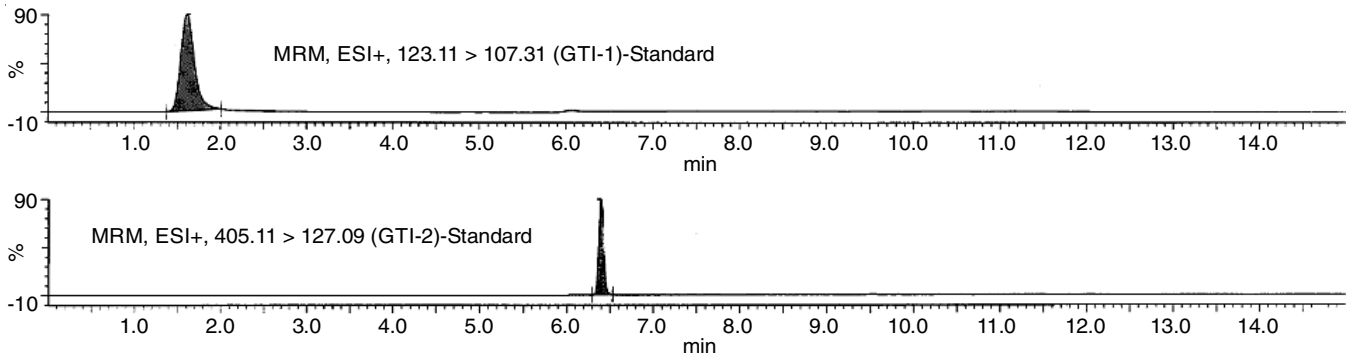


Fig. 4. Standard chromatograms of GTI-1 and GTI-2

Precision: The precision of the method was carried out by analyzing six replicated injections of standard solutions at 2.5 ppm considering the working concentrations of drug substance and the results are summarized in Table-3. The %RSD for GTI-1 and GTI-2 as part of method precision were found to be 3.16 and 3.03, respectively. Repeatability of the method was determined by analyzing six individual sample preparations, spiked with GTIs at 2.5 ppm of the sample concentration by the different analysts using different columns and systems on different days for assessing the method ruggedness. Results obtained are summarized in Table-4. The %RSD for GTI-1 and GTI-2 as part of ruggedness (intermediate precision) were found to be 2.09 and 3.03, respectively. The overall percentage relative standard deviation obtained for GTI-1 and GTI-2 were 3.11 and 2.89, respectively.

TABLE-3
METHOD PRECISION

Sample	GTI-1 (ppm)	GTI-2 (ppm)
1	2.3	2.4
2	2.4	2.6
3	2.4	2.5
4	2.4	2.5
5	2.3	2.4
6	2.5	2.5
Average	2.38	2.48
%RSD	3.16	3.03

TABLE-4
RUGGEDNESS

Sample	GTI-1 (ppm)		GTI-2 (ppm)	
	MP	IP	MP	IP
1	2.3	2.5	2.4	2.5
2	2.4	2.4	2.6	2.6
3	2.4	2.5	2.5	2.5
4	2.4	2.5	2.5	2.4
5	2.3	2.4	2.4	2.5
6	2.5	2.5	2.5	2.4
Average	2.38	2.47	2.48	2.48
%RSD	3.16	2.09	3.03	3.03

Specificity: Blank and spiked sample solutions were prepared at a concentration of about 0.01 mg/mL in the diluent and injected in scan mode. GTI-1, Telbivudine and GTI-2 were well resolved with retention times of 1.76, 3.81 and 6.62 min, respectively. GTI-1 and GTI-2 has shown *m/z* peak $[M+H]^+$ at 123 and 405, respectively. Results obtained are summarized in Table-5 and representative chromatograms are shown in Figs. 5 and 6.

Linearity and range: By diluting standard stock solutions of 2.5 ppm with diluent and analyzing the seven level solutions ranging from quantification limit to 150%, the linearity of the approach was evaluated for GTI-1 and GTI-2. The calibration curve was plotted against concentration and peak area. The intercept, slope and coefficient of correlation were evaluated

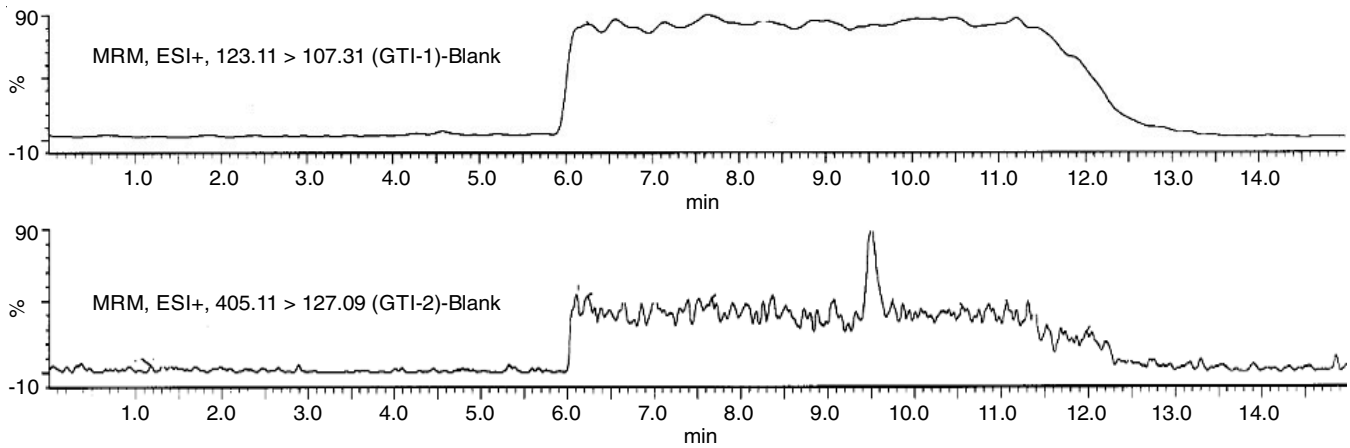


Fig. 5. Blank of GTI-1 and GTI-2

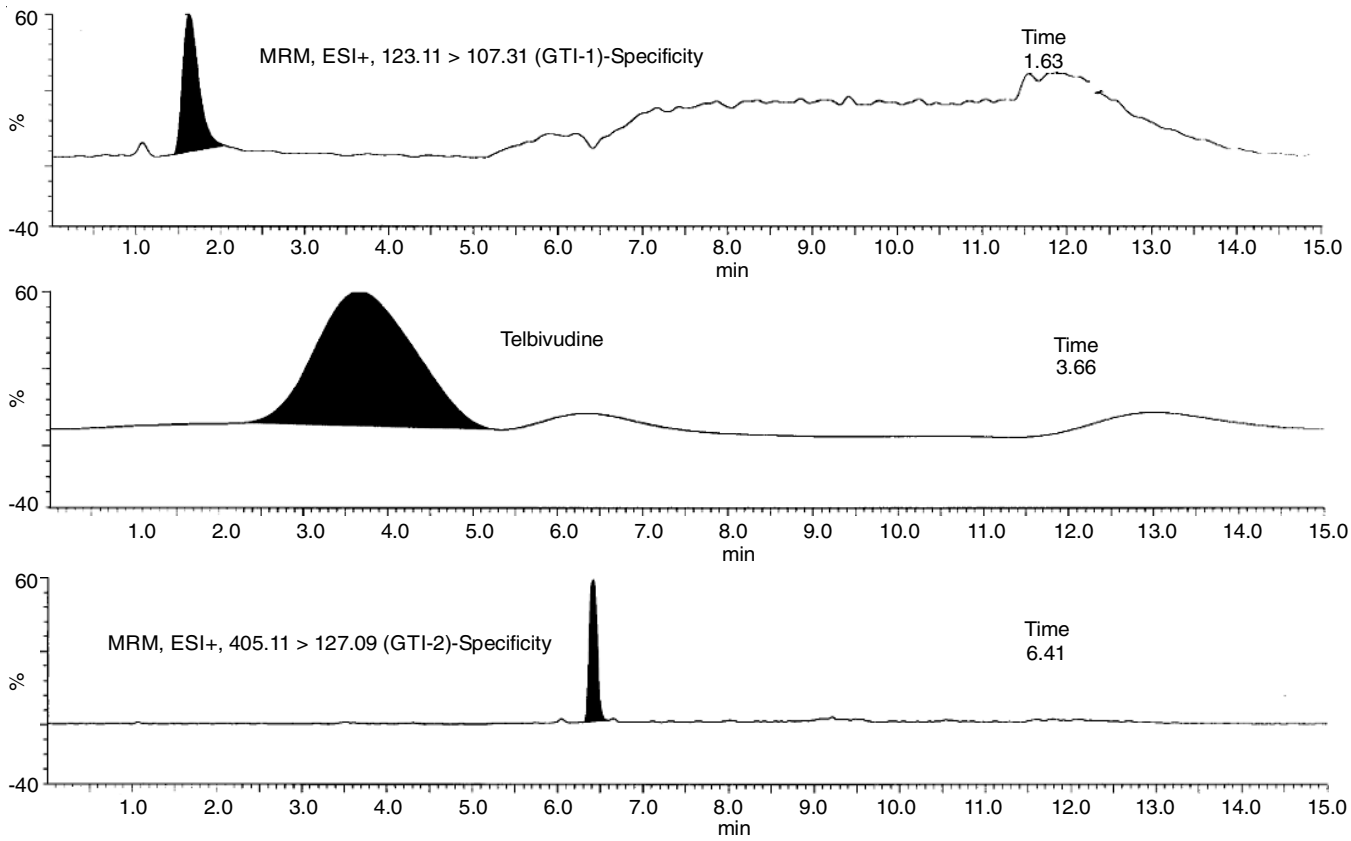


Fig. 6. Specificity chromatogram of GTI-1, Telbuvidine and GTI-2

TABLE-5
SPECIFICITY OF TELBIVUDINE AND ITS GENOTOXIC IMPURITIES

Name of the component	Retention time (min)	Observed mass $(M+H)^+$
GTI-1	1.76	123
Telbivudine	3.81	241
GTI-2	6.62	405

from the statistical regression analysis. The correlation coefficient for GTI-1 and GTI-2 for the above specified concentrations was found to be 0.999. Results obtained are summarized in Table-6.

Accuracy: The experiment was performed by spiking 50%, 100% and 150% of GTIs into the Telbivudine sample solution of 2.5 ppm. For each level, samples were prepared in triplicate. The % recoveries were calculated and found to be in the range of 95.1 to 105.1 for GTI-1 and 97.3 to 103.3 for GTI-2. The % RSD were found to be in the range of 1.15-3.18 for GTI-1 and 0.26-1.61 for GTI-2.Results obtained are summarized in Table-7 and representative chromatograms are shown in Fig. 7.

Detection limit (DL) and quantification limit (QL): The detection limit was established by calculating the S/N (signal-to-noise) ratio and by injecting known concentration of GTIs standard solutions and comparing the test results with blank sample solution, thereby establishing the lowest detection limit for the analyte. Based on the detection limit, roughly three folds of the detection limit solutions were prepared and analyzed

TABLE-6
LINEARITY OF TELBIVUDINE GENOTOXIC IMPURITIES

Level	GTI-1		GTI-2	
	Concentration	Area	Concentration	Area
LOQ	2.513904E-01	12751	2.528542E-01	1497
25	6.284760E-01	33964	6.321355E-01	3689
50	1.256952E+00	72344	1.264271E+00	6995
75	1.885428E+00	101520	1.896407E+00	10884
100	2.513904E+00	135876	2.528542E+00	14953
125	3.142380E+00	169985	3.160678E+00	19134
150	3.770856E+00	210144	3.792813E+00	22996
Slope	55069.7472		6107.4255	
Intercept	-581.4727		-353.0597	
CC	0.9994		0.9994	
R.Square	0.9988		0.9988	

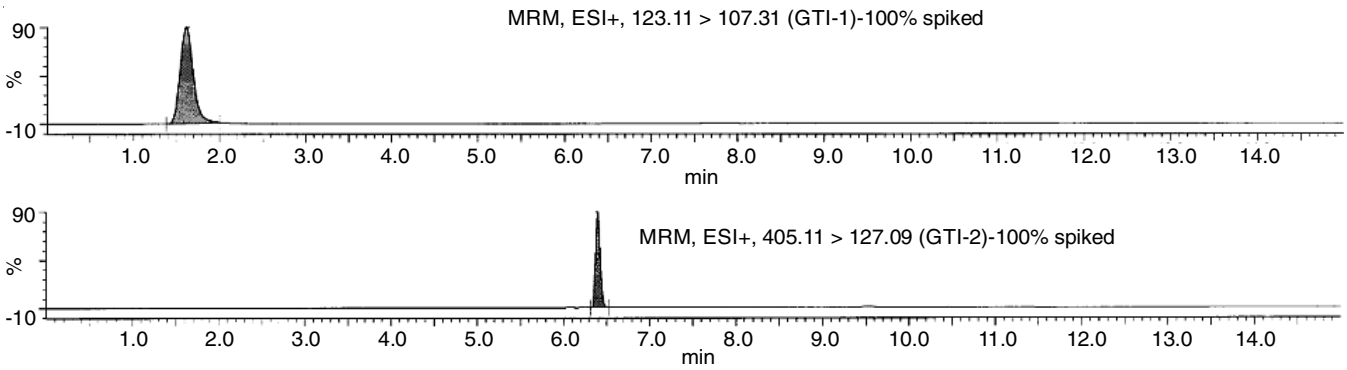


Fig. 7. Spiked sample chromatogram of GTI-1 and GTI-2

TABLE-7
ACCURACY OF TELBIVUDINE GENOTOXIC IMPURITIES

Level (%)	GTI-1		GTI-2	
	% Recovery	%RSD	% Recovery	%RSD
LOQ	103.1	3.18	103.3	1.39
	105.1		100.6	
	98.7		101.1	
50	97.2	1.15	99.7	1.61
	95.8		96.7	
	95.1		97.4	
100	98.5	1.61	97.9	0.53
	95.9		98.4	
	98.8		97.3	
150	96.0	1.19	98.8	0.26
	95.3		98.6	
	97.6		98.3	

for the determination of quantification limit. detection limit were found to be 0.07 (S/N: 8.1) for GTI-1 and 0.09 (S/N: 6.5) for GTI-2. Quantification limit of GTI-1 and GTI-2 were 0.22 (S/N: 27) and 0.27 (S/N: 21), respectively. Results obtained are summarized in Table-8 and representative chromatograms are shown in Figs. 8 and 9.

TABLE-8
SUMMARY OF LOD AND LOQ OF
TELBIVUDINE GENOTOXIC IMPURITIES

Name of the component	Signal to noise ratio	Detection limit (DL) (ppm)	Signal to noise ratio	Quantification limit (QL) (ppm)
GTI-1	8.1:1	0.07	27:1	0.22
GTI-2	6.5:1	0.09	21:1	0.27

Precision at LOQ: Precision at LOQ was established for GTI-1 and GTI-2 (Table-9) and the percentage relative standard deviation for the peak areas of GTI-1 and GTI-2 were found to be 1.7 and 2.2, respectively.

TABLE-9
SUMMARY OF PRECISION AT LOQ

Sample	GTI-1	GTI-2
1	18892	2187
2	18403	2256
3	18832	2276
4	19216	2298
5	18448	2299
6	18587	2194
Average peak area	18729.7	2251.7

Solution stability: Standard solutions, controlled sample and sample spiked with GTIs at 2.5 ppm was prepared and kept at room temperature as well as in the refrigerator at 2-8 ºC. The results are summarized in Table-10. The above results indicate that GTI-1 and GTI-2 in standard solution, the sample solution and spiked solution were stable upto 24 h at 2-8 ºC as well as at room temperature.

Application: The newly developed and validated method was applied to as such formulation samples. Recovery studies were also conducted by spiking GTI-1 and GTI-2 into the formulation samples and chromatograms in Fig. 10. The results related to drug product applications are presented in Table-11. From Table-11, it may be inferred that GTI-1 and GTI-2 were absent in formulation samples. From the recovery experiments, average recovery values were found to be 97.3 % and 97.0 %

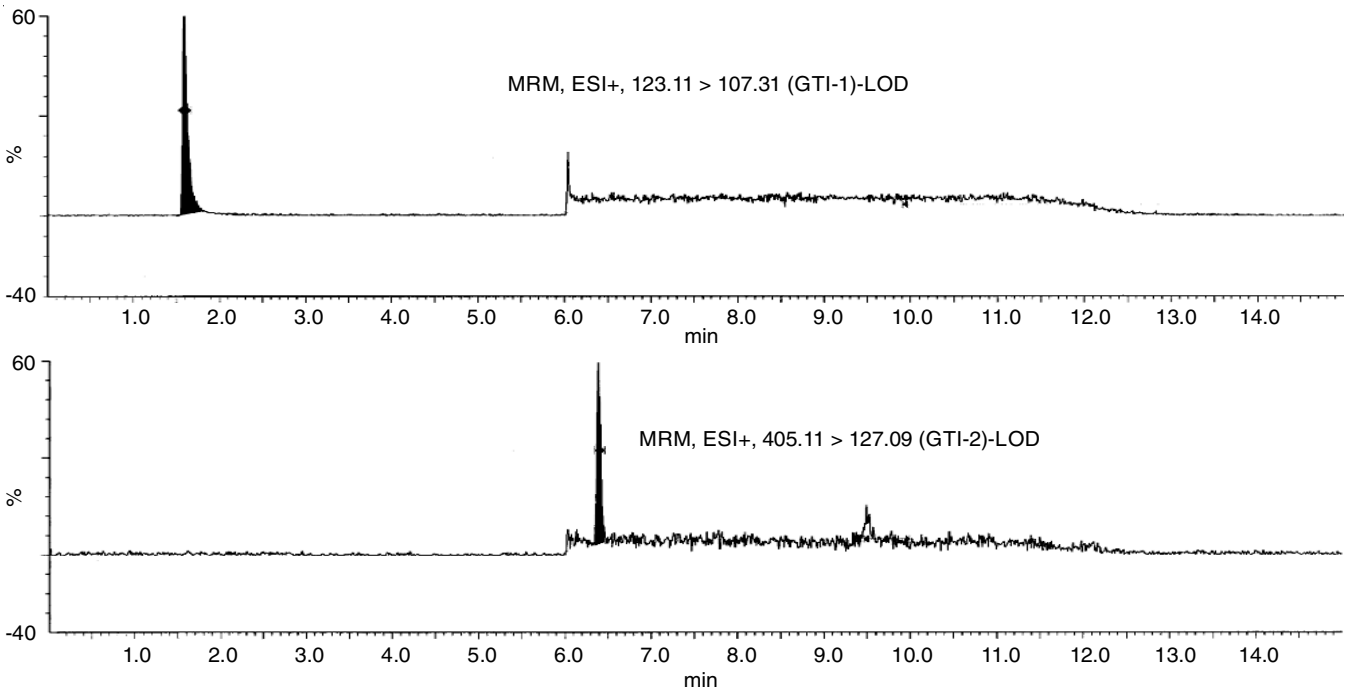


Fig. 8. LOD/DL solution of GTI-1 and GTI-2

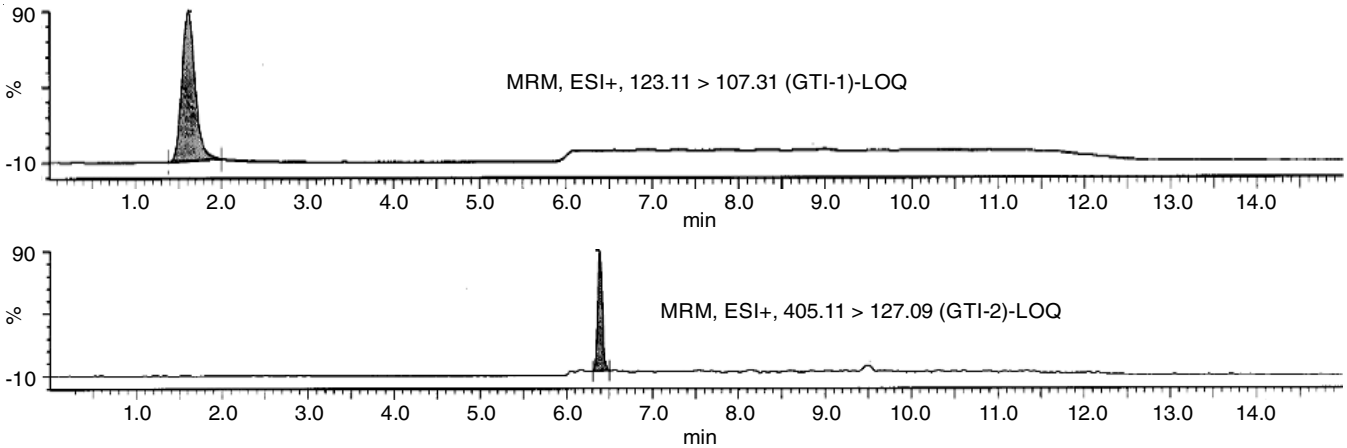


Fig. 9. LOQ/QL solution of GTI-1 and GTI-2

TABLE-10
SOLUTION STABILITY STUDY AT ROOM TEMPERATURE (24-26 °C) AND 2-8 °C

Component	Temperature conditions	Solutions	Initial (ppm)	After 24 h (ppm)	%RSD
GTI-1	Room temperature (24-26 °C)	Standard	2.5	2.5	0.0
		Sample	ND	ND	NA
		Spiked	2.5	2.4	4.0
	2-8 °C	Standard	2.5	2.5	0.0
		Sample	ND	ND	NA
		Spiked	2.4	2.4	0.0
GTI-2	Room temperature (24-26 °C)	Standard	2.4	2.4	0.0
		Sample	ND	ND	NA
		Spiked	2.5	2.5	0.0
	2-8 °C	Standard	2.5	2.5	0.0
		Sample	ND	ND	NA
		Spiked	2.5	2.5	0.0

ND: Not detected. The above results indicate that GTI-1 and GTI-2 in standard solution, sample solution and spiked solution were stable upto 24 h at 2-8 °C as well as at room temperature.

TABLE-11
RESULTS OF APPLICATION

Parameter	GTI-1	GTI-2
Formulation control sample	BDL	BDL
% Accuracy (average recovery of GTI at 100% level in Telbivudine drug product)	97.3	97.0

BDL: Below detection limit

for GTI-1 and GTI-2, respectively. Hence, it is concluded that the present developed method is effective in the trace determination of two potential genotoxic impurities in Telbivudine drug product.

Conclusion

The main goal of this study is to find a way to measure the amount of genotoxic impurities (GTIs) in Telbivudine using RP-UPLC and a mass analyzer which is extremely new, selective, fast, sensitive, accurate, linear, rugged, and reliable. The ICH guidelines were followed to validate the developed and optim-

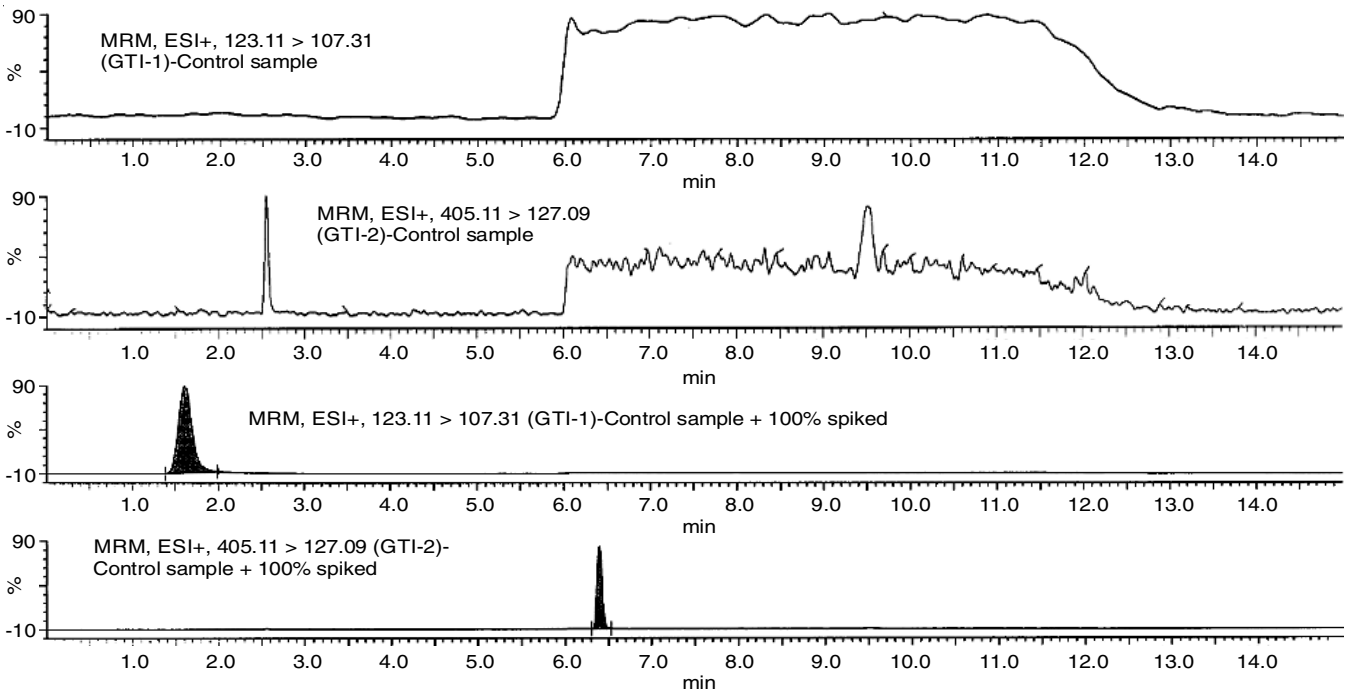


Fig. 10. Control sample chromatogram of GTI-1 and GTI-2

ized UPLC-MS/MS method. The %RSD of precision indicates the method is highly precise in reproducibility. The retention time of the peaks indicates that there is no interference and the GTIs are well separated from the main peak indicating specificity. The values derived from linear least square regression reveal a good correlation between concentrations and areas. The % recovery and %RSD from triplicate samples indicate the diluent selected is more appropriate for extracting the GTIs from the drug substance. The results obtained in this study demonstrated that the developed method is precise, rugged, specific, linear, accurate and sensitive for the determination of GTIs in Telbivudine drug. The stability data of the method indicates the present method can be effectively used for routine development analysis, quality control testing, thereby the method is suitable for the intended use.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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