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Quantitative Determination of Trace Levels of Nitromethane and Its Analog Impurities by Using HS-GC-MS

Siva Krishna Nachaka[1]*, Ramesh Babu Kodavati [1] , Gopal Vaidyanathan [1] , Sattibabu Paila [1] , Narmada Palnati[1]

1.Natco Pharma Limited, Natco Research Centre, B-13, Industrial Estate, Sanathnagar, Hyderabad, Telangana, India - 500018.

ABSTRACT

A specific HS-GC-MS method has been developed, optimized and validated for the determination of five genotoxic impurities namely Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane in TRB-G, intermediate of Trabectedin drug substance. Chromatographic separation of five genotoxic impurities was achieved on Capillary GC column (Rtx-1701. Fused silica capillary column; 30 m length; 0.25mm internal diameter. coated with 14% Cyanopropylphenyl and 86% dimethyl polysiloxane stationary phase of 1.0 µm film thickness) and passing helium carrier gas with Electron Impact ionization (EI) in Selective Ion Monitoring (SIM). The mass fragments (m/z) were selected for the quantification of m/z-30 for Nitromethane, m/z-29 for Nitroethane, m/z-43 for 2-Nitropropane and 1-Nitropropane and m/z-57 for 1-Nitrobutane. The performance of the method was assessed by evaluating the specificity, linearity, sensitivity, precision and accuracy experiments. The established limit of detection values for the genotoxic impurities were in the range of 0.25 µg/g – 0.58 µg/g and limit of quantification values were in the range of 0.75 µg/g – 1.75 µg/g. This developed method was found to be linear with correlation coefficient is greater than 0.999. The average recoveries for the accuracy were in the range of 89.4–106.8%. Developmental and validation experiments were discussed in detail in this paper.

Keywords: Trabectedin (TRB), HS-GC-MS, Genotoxic impurities and Method validation.

*Corresponding Author Email: sivakrishna.sri2008@gmail.com
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INTRODUCTION

Trabectedin (ET-743, Yondelis®) is a novel marine origin antineoplastic alkaloid with a unique mechanism of action [1]. The active substance Trabectedin, a tetrahydroisoquinoline alkaloid that binds to the minor groove of DNA [2]. It is a natural product originally isolated from the Caribbean Sea squirt, Ecteinascidia turbinata and is currently manufactured by total synthesis. Trabectedin (TRB) is licensed by the Spanish pharmaceutical drug company, Pharma Mar and co-developed by Johnson & Johnson Pharmaceutical Research and Development, L.L.C., pursuant to a licensing agreement with Pharma Mar. Trabectedin is the first anticancer marine-derived drug to be approved by the European Union. In 2007, Trabectedin obtained marketing authorization from the European Commission and in many other countries worldwide for the treatment of patients with advanced soft tissue sarcoma (STS) after failure of anthracyclines and ifosfamide, or for those patients who are unsuitable to receive these agents [3]. Based on the recently reported results of a large phase III study (OVA-301) comparing pegylated liposomal doxorubicin (PLD) alone with a combination of PLD and trabectedin in patients with recurrent ovarian cancer, in 2009 the European Commission granted marketing authorization for Trabectedin combined with PLD for the treatment of patients with relapsed platinum-sensitive ovarian cancer. The results from OVA-301 showed that the combination of trabectedin and PLD improves progression-free survival and overall response rate over PLD alone with acceptable tolerance in the second-line treatment of recurrent ovarian cancer. In addition, an enhanced activity of Trabectedin combined with PLD was observed in platinum sensitive patients, especially in those with a platinum-free interval ranging from 6 to 12 months. Overall, Trabectedin-induced toxicities are mainly hematological and hepatic, with grade 3/4 neutropenia and thrombocytopenia observed in approximately 50% and 13% of patients, respectively and grade 3/4 elevation of liver aminotransferases observed in 40-50% of patients treated with Trabectedin. Current efforts are focused on the evaluation of the role of Trabectedin in prolonging the platinum-free interval and the identification of predictive factors for patients treated with Trabectedin as well as in the development of new Trabectedin-based combinations.

Trabectedin used for the treatment in cancer/tumors (unspecified), gastric cancer, ovarian cancer, pediatric indications, sarcoma, and solid tumors [4-7]. It interacts with the minor groove of DNA and alkylates guanine at the N2 position, which bends towards the major groove. In this manner, it is thought that the drug affects various transcription factors involved in cell proliferation, particularly via the transcription-coupled nucleotide excision repair system and blocks the cell cycle at the G2 phase, while cells at the G1 phase are most sensitive to the drug. It also inhibits overexpression of

the multidrug resistance-1 gene (MDR-1) coding for the P-glycoprotein that is a major factor responsible for cells developing resistance to cancer drugs. The agent is also thought to interfere with the nucleotide excision repair pathways of cancer cells, suggesting that it could be effective in the treatment of many cancer types including melanoma and sarcoma, as well as lung, breast, ovarian, endometrial and prostate cancers. The chemical name of Trabectedin is (1′*R*,6*R*,6a*R*,7*R*,13*S*,14*S*,16*R*)-6′,8,14-trihydroxy-7′,9-dimethoxy-4,10,23-trimethyl-19-oxo-3′4′,6,7,12,13,14,16-octahydrospiro[6,16-epithiopropano-oxymethano)-7,13-imino-6aH-1,3-dioxolo[7,8] isoquino[3,2-b][3]benzazocine-20,1′(2′H)-isoquinolin]-5-yl acetate corresponding to the molecular formula $C_{39}H_{43}N_3O_{11}S$.

Synthesis of drug substances often involves the use of different raw materials and hence, these raw materials and its impurities may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose [8]. These limits generally fall at low mg/mL levels and hence conventional HPLC, GC methods (or final drug substance methods) may not suitable for their determination. Hyphenated techniques like GC–MS and LC–MS combine physical separation capabilities of chromatography (GC or HPLC) with the mass analysis capabilities of mass spectrometry and have high sensitivity and specificity over conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances [9].

Nitro alkanes are irritants to the eyes and the mucous membrane of the respiratory tract. High exposure levels of nitro alkanes cause Anaesthesia and methemoglobinemia [10, 11]. Based on studies in animals, 2-nitropropane and nitromethane are reasonably anticipated to be human carcinogens, and both are listed as International Agency for Research on Cancer Group 2B carcinogens. Nitromethane is one of the toxic nitro compounds included in the FDA's HPHCs list because of its probable carcinogenicity, as indicated in animal, exposure, metabolic, and structure-activity relationship studies[12, 13].

The following impurities Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane are likely present in Trabectedin (TRB) drug substance. In these, Nitromethane is used as a key raw material for the preparation of Trabectedin (TRB) drug substance. The other four are possible isomeric impurities. Based on literature and evaluation by Derek software, these five compounds are found to be mutagenic and carcinogenic. Hence, these genotoxic impurities are limited to a daily dose of 1.5µg/day as per ICH guidelines from the European medical agency [14]. Hence, in order to meet the regulatory agencies requirements, it is essential to develop a sensitive

analytical method. Hence, a gas chromatograph with mass spectrophotometer was chosen which can detect low level determinations for the quantification of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane.

The European Agency for the Evaluation of Medicinal products (EMEA), United States Food and Drug Administration (USFDA) and ICH Q3A/B issued the guidelines and draft guidance on the limitation of genotoxic impurities in pharmaceutical ingredients [15, 16]. Based on these current regulatory guidance's for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5mg/day daily intake of individual impurity. These impurities limit is considered as 7 µg/gm with respect to Trabectedin (TRB) drug substance maximum daily dose (1mg/vial) [17-21]. Appropriate analytical methods were required for the monitored and controlled this genotoxic impurity for the best quality of TRB drug.

In 1968, Hoffmann and Rathkamp were the first to report Nitromethane levels in cigarette smoke by using gas chromatography with an electron capture detector (GC-ECD) in a multi-analyte method for analysis [22]. More recently, Sampson et al., developed a multi-analyte volatile organic compound (VOC) panel that includes nitromethane by using gas chromatography–mass spectrometry (GC–MS) [23]. In 2015, Wang et al. published an analytical method for determining nitro compounds in mainstream cigarette smoke by using gas chromatography and mass spectrometry (GC-GC–MS) [24].

Being a very novel and recently synthesized drug, there are few references for Trabectedin. Through the review of the above reported methods, none of the method was described for the quantification of the five genotoxic impurities (Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane) in TRB drug substance. For the trace level determination of all the five genotoxic impurities in TRB-G, one of the intermediate of Trabectedin drug substance, a single, sensitive and specific GC-EI-MS with selective ion monitoring (SIM) mode method was developed and validated. The chemical structures of Trabectedin, TRB-G intermediate and five impurities are shown in Figures 1a to 1g.

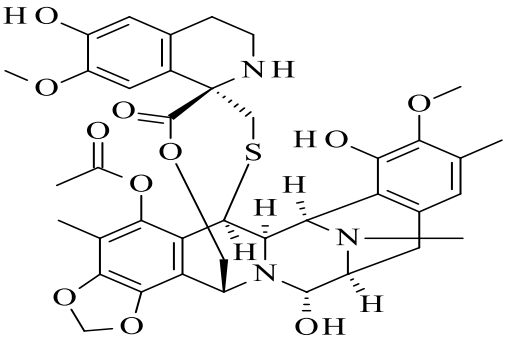


Figure 1a: Chemical structure of Trabectedin API

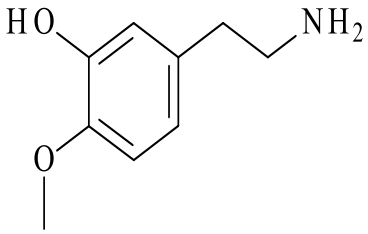


Figure 1b: Chemical structure of TRB/G Intermediate

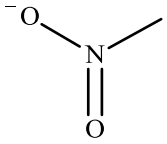


Figure 1c: Nitromethane

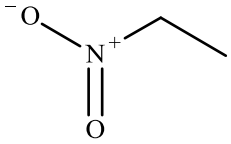


Figure 1d: Nitroethane

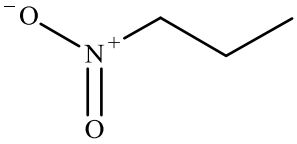


Figure 1e: 1-Nitropropane

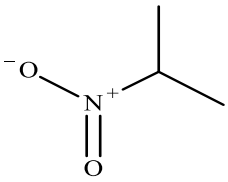


Figure 1f: 2-Nitropropane

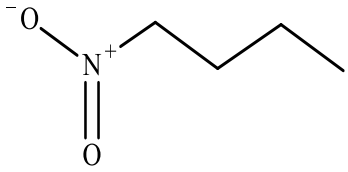


Figure 1g: 1-Nitrobutane

MATERIALS AND METHOD

Chemicals and reagents:

Pure samples of TRB-G were obtained from Chemical research division of NATCO Research Centre (A division of NATCO Pharma Ltd.), Hyderabad, India. Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane, 1-Nitrobutane, Methylene chloride, Methanol, Isopropyl alcohol, Diisopropyl ether, Ethyl acetate and Tetrahydrofuran were procured from Sigma Aldrich, Steinheim, Germany. N-Methyl-2-pyrrolidinone (NMP) (Grade: GC) were procured from Rankem, India.

Preparation of solutions:

Blank solution:

To the headspace vial, added 1.0mL of N-Methyl-2-pyrrolidinone (NMP) and sealed the vial immediately. This sealed vial was used as blank solution.

Standard stock solution:

Weigh accurately about 44 mg each of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane standards into a 50 mL of volumetric flask half-filled with diluent and makeup to volume with diluent and mix well. Transfer 1.5 mL of this solution in to a 25 mL volumetric flask and makeup to the volume with diluent.

Standard solution:

Transfer 1.0 mL of above standard stock solution in to a 50 mL volumetric flask and dilute to volume with diluent and mix well. To the headspace vial, added 1.0mL of standard solution and sealed the vial immediately. This sealed vial was used as Standard solution.

Sample solution:

Accurately weigh and transfer about 150mg of sample into the headspace vial. Add 1.0 ml of N-Methyl-2-pyrrolidinone and seal the vial immediately.

GC-MS Conditions:

The complete experiments were performed on the Agilent GCMS-5977A and GCMS-5977B gas chromatograph equipped with 7890B GC System and 7697A Headspace sampler and data handling system having Mass Hunter solution software. The instrument was run in EI mode. Rtx-1701, (30m × 0.25 mm I.D, 1.0 µm film thickness, Restek, USA) column consists of 14% Cyanopropylphenyl and 86% dimethyl polysiloxane as a stationary phase. Chromatographic method conditions used were as follows (Tables 1-3).

Table 1: Gas chromatograph conditions for GTI analysis

Instrument	Agilent 7890B
Column	Rtx-1701, 30 m x 0.25 mm I.D. x 1.0µm Film thickness
Carrier gas	Helium
Injector temperature (°C)	220°C
Injection type	HS (Headspace) control
Column oven program	40°C (4min) $\xrightarrow{10° C/min}$ 200°C (2min).
Flow rate (mL/min)	0.8
Split ratio	20:1
Run time (min)	22

Table 2: MS Parameters:

Instrument	Agilent GCMS-5977A and GCMS-5977B Single Quad MS
MS transfer line temperature (°C)	250
MS source temperature (°C)	230
Function type	SIM (selective ion monitoring)
Gain factor	5
Timed MS Detector:	
The MS must be ‘ Detector Off ’ after 13.7 minutes	

Table 3: Headspace Parameters:

Oven temperature	:	100°C
Loop Temperature	:	110°C
Transfer line Temperature	:	120°C
Vial equilibration time	:	20 minutes
Injection duration	:	1.0 minutes
GC cycle time	:	30 minutes
Pressure equilibration time	:	0.2 minutes
Loop equilibrium time	:	0.05 minutes
Fill pressure	:	14.2 psi
Final loop pressure	:	2 psi
Vial size	:	20 mL
Shaking vials while in oven	:	Middle (Level-5 or 71 shakes/min)

RESULTS AND DISCUSSION:

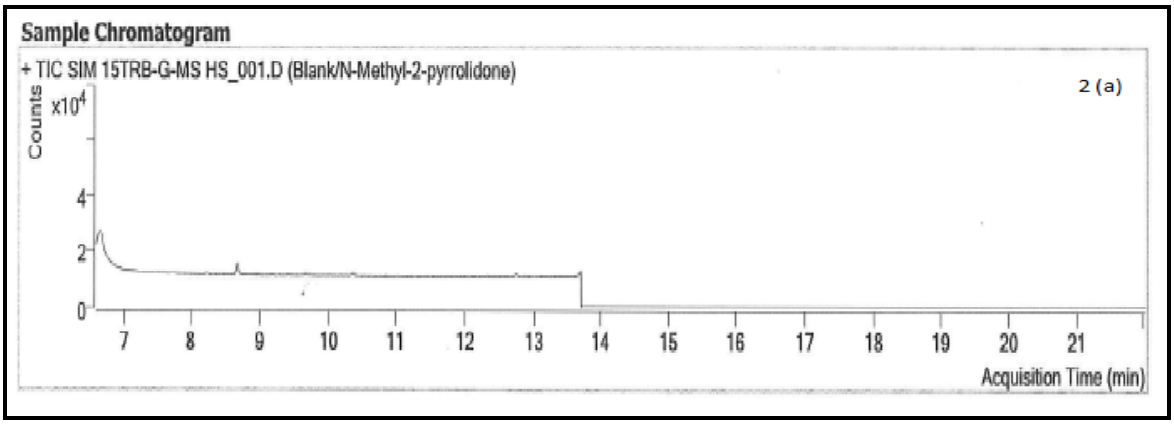
Method validation:

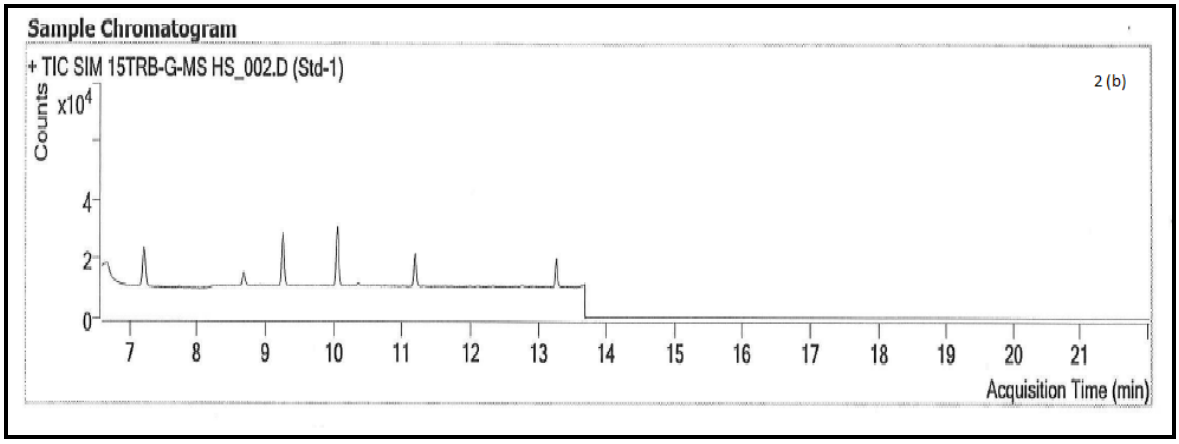
The developed method was validated as per the ICH guidelines [25] for the determination of the contents of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane in TRB-G intermediate. Individually in terms of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (system precision, method precision and intermediate precision) and robustness and system suitability.

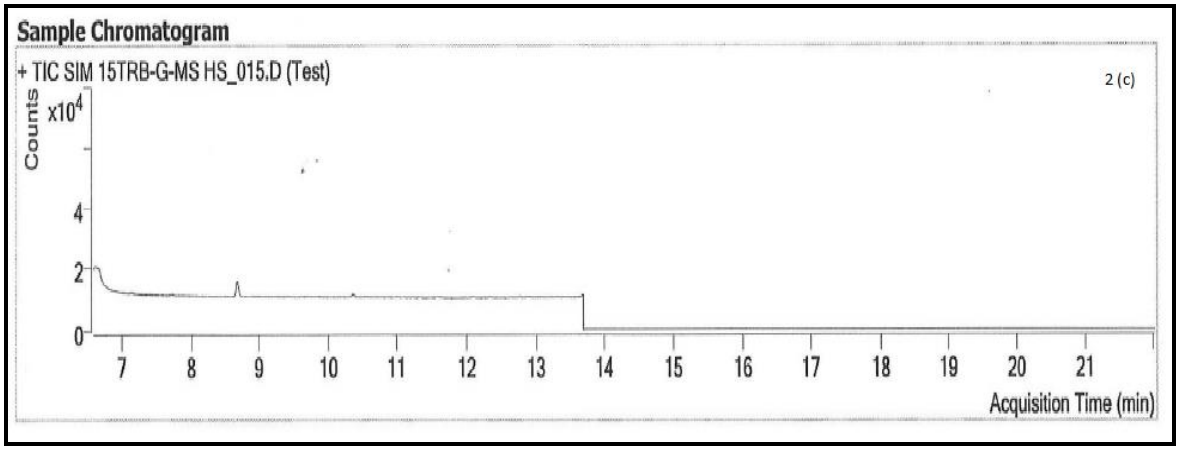
Specificity:

The specificity of the developed GC-MS method was indicated by showing the m/z peaks in the method as 30 for Nitromethane, 29 for Nitroethane, 43 for 2-Nitropropane and 2-Nitropropane and 57 for 1-Nitrobutane. Specificity is the ability of the method to measure the analyte response in presence of all impurities (Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane) in TRB-G. The specificity of the developed GC-MS method was verified in presence of residual solvents like Methylene chloride, Methanol, Isopropyl alcohol, Diisopropyl ether, Ethyl acetate and Tetrahydrofuran which were used in the TRB-G process. These solvents and five analytes were injected individually to confirm retention times. TRB-G sample solution (Control sample), TRB-G drug substance spiked with Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane at specification level (Spiked Sample) and TRB-G spiked with Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane and all other known residual solvents at specification level (All Spiked Sample), Blank and Standard solutions were injected into GC-MS to confirm any co-elution of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane peaks with each other and with any other known residual solvents. The specificity experiment typical GC-MS Chromatograms of Blank, Standard,

Control sample, Spiked sample and All spiked sample are shown in Figures 2 (a) to (e). Based on evaluation of specificity studies, it was concluded that the Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane peaks are well separated from each other as there is no other solvent co-elution indicated that the method is selective and specific for five analytes in TRB-G intermediate.







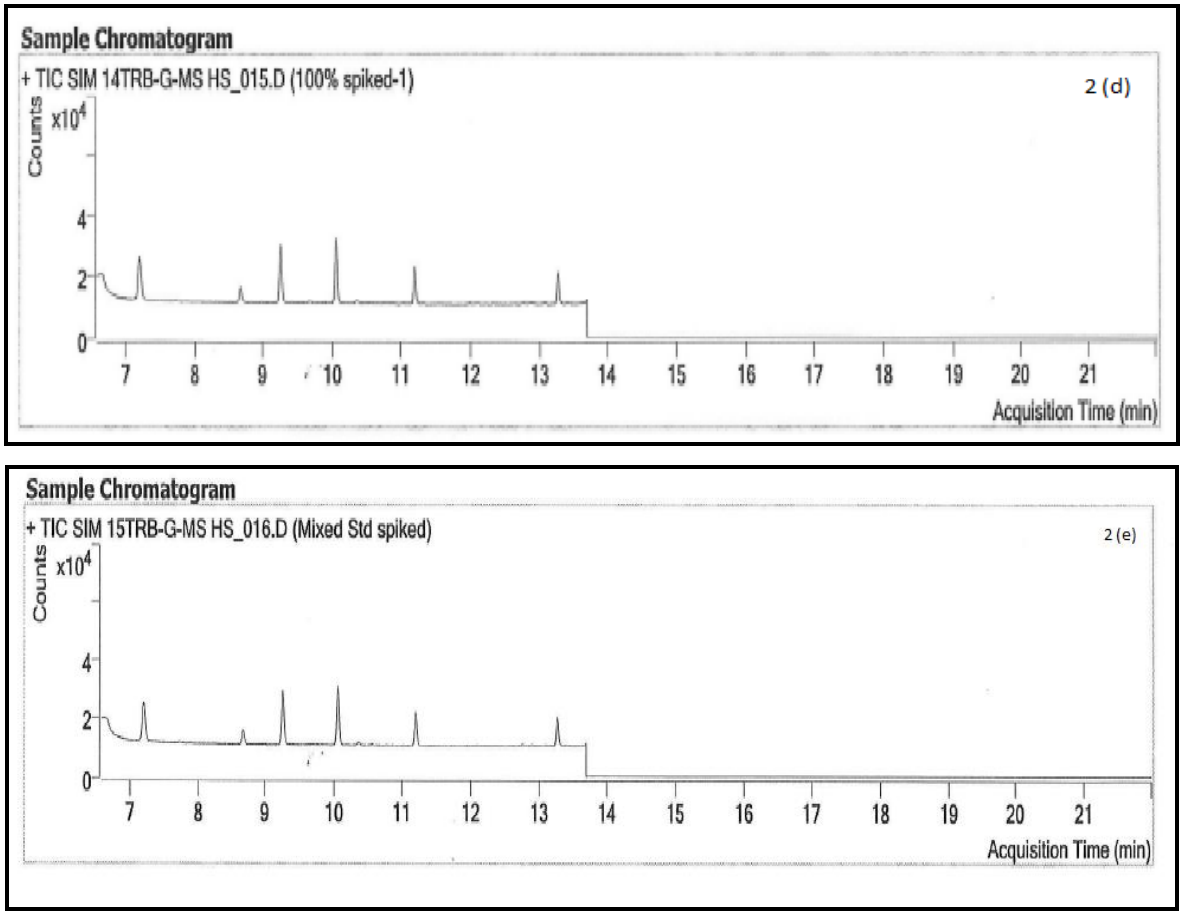


Figure 2: Typical GC-MS chromatograms of a) Blank solution, (b) Standard solution, (c) TRB-G (control sample), (d) TRB-G spiked with GTI's (spiked sample) and (e) TRB-G spiked with GTI's including all residual solvents (all spiked sample)

Limit of detection and limit of quantification:

In this method, Specification level standard solution was injected in to GC-MS and S/N ratios for all analytes were recorded. Based on these values, the LOD and LOQ values of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane were predicted. At LOQ level S/N ratio was > 10 and LOD level S/N ratio was > 3 for all analytes. Each predicted concentration was verified for precision by preparing the solutions at about these predicted concentrations and injected each solution six times into the GC-MS. The details of the précised LOD and LOQ values are shown in Table 4. The overlaid GC-MS chromatogram LOQ solution are shown in Figure 3 (a).

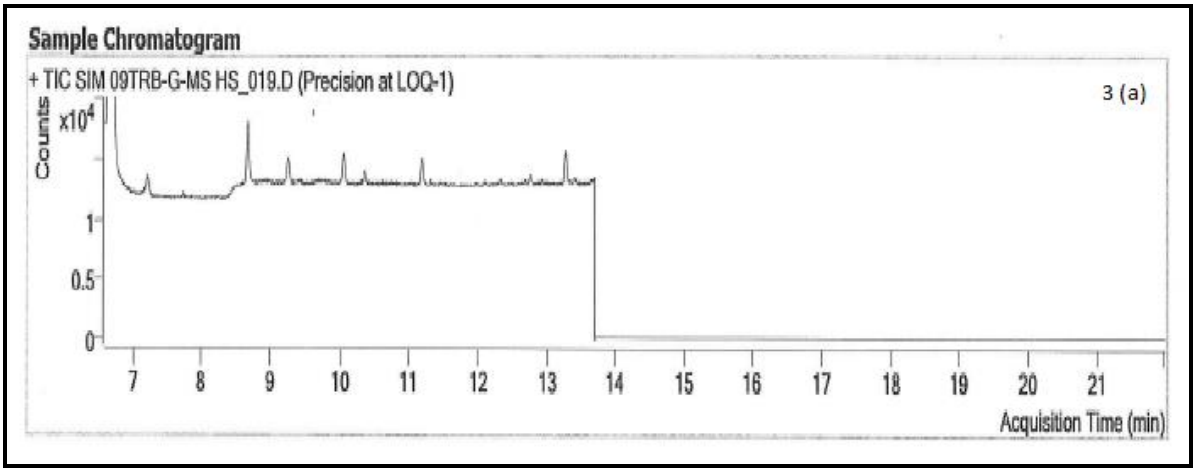


Figure 3: The overlaid GC-MS chromatogram LOQ solution

Linearity:

The linearity of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane was satisfactorily done. A series of solutions were prepared across the range concentrations were studied in the range of LOQ to 150% of the specification level were prepared and injected each in duplicate injections into GC-MS. Statistical data like slope, intercept, STEYX and correlation coefficient were established by using the peak area response versus concentration data. The derived correlation coefficients were in the range of 0.9975–0.9985 indicating the best fitness of the linearity curves of the developed method. The calculated statistical results are shown in Table 4.

Accuracy:

Standard addition experiments were conducted in triplicate preparations (*i.e.* TRB-G sample solutions were prepared in triplicate by spiking with Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane) to determine accuracy of the methods at LOQ level, 50% level (3.5ppm), 100% level (7ppm) and 150% level (10.5ppm). In the accuracy experiment, TRB-G sample solutions (control sample) were prepared without spiking any impurity in triplicate and injected into GC-MS. Further, TRB-G sample solutions (spiked sample) were prepared in triplicate by spiking with all the impurities and injected into GC-MS. Control samples, Spiked samples were analysed and the percentage recoveries were calculated. The average % recovery values of four levels (LOQ, 50%, 100% and 150% levels) for twelve determinations for 98.4 (Nitromethane), 100.7 (Nitroethane), 98.3 (2-Nitropropane), 96.7 (1-Nitropropane) and 96.6 (1-Nitrobutane). The accuracy experiment results are reported in Table 5

Table 4: LOD, LOQ and Linearity experiments results

Statistical parameters	Results				
	Nitromethane	Nitroethane	2-Nitropropane	1-Nitropropane	1-Nitrobutane
Correlation coefficient	0.9999	0.9999	1.0000	0.9999	0.9999
Concentration range (ppm)	0.75 - 10.58	0.75 - 10.60	0.74 - 10.41	1.20 - 10.59	1.75 - 10.49
Calibration points	7	7	7	7	6
Intercept	880.95	755.36	829.93	701.82	393.45
Slope(S)	5073.7990	5301.4931	6242.1185	3079.3332	1130.4516
STEYX	361.3130	370.9088	294.1214	213.4616	60.6949
LOD (ppm)	0.25	0.25	0.25	0.40	0.58
LOQ (ppm)	0.75	0.75	0.75	1.20	1.75
Precision at LOD level (% R.S.D)	3.6	13.4	8.3	11.0	2.9
Precision at LOQ level (% R.S.D)	2.6	5.1	2.7	6.3	3.3

Table 5: Accuracy experiment results

Identification	**Nitromethane**				**Nitroethane**				**2-Nitropropane**			
Control sample	ND				ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	0.746	3.53	7.06	10.58	0.749	3.53	7.07	10.60	0.741	3.47	6.94	10.41
*Found (µg/g)	0.770	3.46	6.85	10.07	0.765	3.62	7.06	10.45	0.757	3.43	6.74	9.89
Recovery (%)	103.2	98.1	97.1	95.2	102.1	102.4	99.9	98.6	102.2	98.8	97.1	95.0
% RSD	3.9	1.8	1.1	1.3	3.3	3.9	0.4	1.3	3.2	2.8	1.0	1.4
Identification	**1-Nitropropane**					**1-Nitrobutane**						
Control sample	ND					ND						
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)		LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)			
*Added (µg/g)	1.203	3.53	7.06	10.59		1.745	3.50	7.00	10.49			
*Found (µg/g)	1.240	3.39	6.68	9.80		1.863	3.34	6.61	9.38			
Recovery (%)	103.1	96.1	94.6	92.6		106.8	95.5	94.5	89.4			
% RSD	3.3	4.1	1.4	2.0		0.9	4.6	1.5	2.5			

*Average of three replicates.

ND: Not Detected.

Precision:

The precision was the study of the method using repeatability (Method precision) and reproducibility (Ruggedness). The performance of the method was evaluated with replicate injections of standard and sample solutions. Standard solution was analyzed by injecting six times for checking the performance of the GC-MS system under the test conditions on the day tested (System Precision). The relative standard deviation results achieved for the system precision experiment were reported in Table 6. Repeatability (Method Precision) experiment was performed by prepared six sample solutions were using single batch of TRB-G spiked with Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane about known concentration (7ppm) level and injected into GC-MS. The relative standard deviation for the content results of the Method precision experiment were reported in Table 6.

Reproducibility (Method precision) (µg/g)					
1	6.88	7.09	6.77	6.76	6.74
2	6.92	7.07	6.80	6.73	6.57
3	6.76	7.03	6.66	6.56	6.54
4	6.82	7.06	6.71	6.57	6.39
5	6.90	7.08	6.70	6.66	6.45
6	6.79	6.99	6.63	6.52	6.19
Average	6.84	7.05	6.71	6.63	6.48
STDEV	0.06	0.04	0.06	0.10	0.19
%RSD	0.9	0.6	0.9	1.5	2.9
Reproducibility (Intermediate Precision) (µg/g)					
1	7.01	6.84	6.57	6.80	6.74
2	6.90	6.78	6.46	6.56	6.24
3	6.73	6.73	6.42	6.55	6.35
4	7.05	7.25	6.71	6.94	7.09
5	6.87	6.95	6.59	6.83	6.81
6	7.19	7.11	6.80	7.17	7.33
Average	6.96	6.94	6.59	6.81	6.76
STDEV	0.16	0.20	0.14	0.24	0.42
%RSD	2.3	2.9	2.1	3.5	6.2
Overall statistical data(n=12)					
Average	6.90	7.00	6.65	6.72	6.62
STDEV	0.13	0.15	0.12	0.20	0.34
%RSD	1.9	2.2	1.9	2.9	5.1

The intermediate precision was the inter-day variation (ruggedness) was defined as the degree of reproducibility obtained by following the same procedure as mentioned for method precision experiment. Ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in Method precision experiment) by spiking Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane to TRB-G drug substance and injected into different column, different instrument and different analyst on different

days. The obtained precision (System precision, Method precision and Intermediate precision) experiment results are shown in Table 6.

Table 6: Statistical Data of Precision experiment

Repeatability (System precision) Area	Nitromethane	Nitroethane	2-Nitropropane	1-Nitropropane	1-Nitrobutane
1	40505	43517	50768	26838	10570
2	39917	42616	49391	25886	9981
3	40217	42214	49035	25823	9974
4	40099	41000	48587	25502	9722
5	41505	42214	49873	26502	10022
6	39627	40058	46973	24850	9371
Average	40312	41937	49105	25900	9940
STDEV	654.24	1226.34	1284.98	708.42	394.40
%RSD	1.6	2.9	2.6	2.7	4.0

Robustness:

Robustness of the method was evaluated by deliberately altering the method conditions from original method parameters and verifying compliance to the system suitability parameters. The impact of variation of column oven temperature and flow rate of carrier gas on system suitability was conducted. In robustness verification of test method, one parameter changed while keeping the other unchanged from actual parameter. The study was carried out with respect to Column flow variation of carrier gas initial flow rate ±10% and column oven initial temperature and ramp temperature ± 2°C and Headspace vial oven temperature ± 5°C as follow listed in Table 7a and Table 7b. Results of peak areas for Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane are summarized in Table 8. In each robustness conditions remaining GC-MS conditions are same as per test method.

In each robustness conditions, remaining HS-GC-MS conditions are same as per test method.

Table 7a: Flow variations:-

Column Flow (ml/min)	
As per Methodology	0.80
-10% Flow variation	0.72
+10% Flow variation	0.88

Table 7b: Column Oven, Ramp and Headspace vial temperature variations:-

Column Flow (ml/min)			
As per Methodology	40°C (4min)	10° C/min ⟶	200°C (2min).
-2°C Column Oven and Ramp Temperature variation	38°C (4min)	8° C/min	200°C (2min).

+2°C Column Oven and Ramp	12° C/min	
Temperature variation	42°C (4min)	200°C (2min).
Headspace vial Oven temperature (-5°C):	95°C.	
Headspace vial Oven temperature (+5°C)	105°C.	

Table 8: Robustness experiment results

Robustness condition	**System suitability criteria (% RSD)**				
	Nitromethane	**Nitroethane**	**2-Nitropropane**	**1-Nitropropane**	**1-Nitrobutane**
As per methodology	1.6	2.3	1.8	2.3	2.0
Flow variation					
-10%	0.8	1.0	1.3	1.5	1.9
+10%	1.6	1.7	1.5	1.5	1.3
Temperature variation - Initial Oven and ramp					
-2°C	3.2	3.0	3.3	4.3	5.3
+2°C	1.1	1.1	1.1	0.7	1.1
Headspace vial oven Temperature variation					
-5°C	1.3	0.8	0.8	1.0	0.9
+5°C	0.5	0.4	0.3	0.7	0.8

CONCLUSION

The present study established a well resolved analytical method for the determination of five genotoxic impurities by HS-GC-EI-MS with SIM mode at a very low level. Method validation data demonstrated that the developed method is a simple, sensitive, specific, precise, linear, accur ate, user friendly and cost-effective for the estimation of Nitromethane, Nitroethane, 2-Nitropropane, 1-Nitropropane and 1-Nitrobutane contents in TRB-G, intermediate of Trabectedin drug substance.

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