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A SENSITIVE HEADSPACE METHOD FOR THE DETERMINATION OF 2-BROMOPROPIONIC ACID AND 2-BROMOPROPIONYL CHLORIDE IN TIOPRONIN DRUG SUBSTANCE BY GC-MS

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ABSTRACT
A selective GC-MS method was developed and evaluated for the determination of genotoxic impurities namely, 2-Bromopropionic acid (Br-PrOH) and 2-Bromopropionyl chloride (Br-PrCl) in Tiopronin (TPN) drug substance. The background of the method involves derivatization process, in this process, both analytes (Br-PrOH and Br-PrCl) are converted into Methyl 2-Bromopropionate with methanol in the presence of sulfuric acid and both analytes quantified as Methyl 2-Bromopropionate. Chromatographic separation was achieved on Zebron ZB-624 column (30 m x 0.32 mm, 1.8 µm), that contains 6% of cyanopropylphenyl and 94% of dimethylpolysiloxane as stationary phase by passing helium as carrier gas. Mass fragment m/z-107 was selected as quantification ion and m/z-87 was selected as qualifier ion for the determination of Methyl 2-Bromopropionate. The performance of the method was assessed by evaluating the specificity, linearity, sensitivity, precision and accuracy experiments. The method was specific and precise for 2-Bromopropionic acid and 2-Bromopropionyl chloride including other mass fragments exits from Tiopronin. The limit of detection (LOD) and limit of quantification (LOQ) values for Methyl 2-Bromopropionate was 0.004 µg mL-1 and 0.012 µg mL-1 respectively. The correlation co-efficient value for linearity experiment was 0.9992. The average recovery was in the range of 97.0% to 102.9%. The results proved that the method is suitable for the determination of Br-PrOH and Br-PrCl contents as Methyl 2-Bromopropionate in Tiopronin drug substance and method can be successfully applied for the quality control analysis.

KEYWORDS: Gas chromatography, Tiopronin, Genotoxicity, Derivatization, Quantification.

1. INTRODUCTION

Chemically, Tiopronin (TPN) is known as 2-(2-Sulfanylpropanoylamino) acetic acid. The molecular formula of Tiopronin is $C_5H_9NO_3S$ and the molecular weight is 163.19. Tiopronin (TPN) is a prescription thiol drug, used to control the rate of cystine precipitation and excretion in the disease cystinuria.[1-2] It is also recommended for the treatment of Wilson's disease (an overload of copper in the body), arthritis[3-4] and use as a stabilizing agent for metal nanoparticles to preventing coagulation.[5] Tiopronin falls under the classification of an orphan drug on the basis of the rarity disorder. Tiopronin is marked under the trade name of Thiola. In the synthesis process of Tiopronin drug substance, 2-Bromopropionic acid (Br-PrOH) and 2-Bromopropionyl chloride (Br-PrCl) are used as raw materials. As per the toxicological threshold concern (TTC) approach, these genotoxic impurities (Br-PrOH and Br-PrCl) should be less than 10 µg g^{-1} on based on the extreme intake of Tiopronin is 1.0 g / day. As a part of good manufacturing practices, Br-PrOH and Br-PrCl raw materials might be carry through the manufacturing process of Tiopronin (TPN) drug substance. Usually brominated impurities [Br-PrOH and Br-PrCl] are cancer suspicious agents and falls under the genotoxic category and these brominated impurities should be monitored and quantified with suitable analytical methods in Tiopronin drug substance. The European Agency for the Evaluation of Medicinal products (EMEA) (2006); United States Food and Drug Administration (USFDA) (2008) and ICH Q3A/B (2006) issued the guidelines and draft guidance on the limitation of genotoxic impurities in pharmaceutical ingredients.[6-7]

In the available literature, few reports have been published for the quantification of 2-Chloropropionic acid by different analytical techniques. A head space GC-MS was used for the estimation of 2-Chloropropionic acid in Iopamidol drug substance.[8] A capillary electrophoresis method with diode array detector was reported for the resolution of racemic 2-Chloropropionic acid.[9] From the assessment of the above detailed methods, no any analytical procedure is available for estimating of 2-Bromopropionic acid and 2-Bromopropionyl chloride in drug or drug product from the literature survey. Therefore, it is aimed to develop a sensitive, user-friendly chromatography method for the quantification of Br-PrOH and Br-PrCl in Tiopronin drug substance.

2. MAERIALS AND METHODS

2.1 Instrumentation, reagents and chromatographic conditions

An experimental work conducted on the Agilent GCMS-5977A gas chromatograph equipped with GC sampler 80 auto sampler and MassHunter software. Zebron ZB-624, (30 m x 0.32 mm, 1.8 µm) capillary column coated with 6% cyanopropylphenyl and 94% dimethylpolysiloxane material (Make: Phenomenex). Helium gas (Purity not less than 99.999) was used as carrier gas. The column flow was 1.5 mL/min. Initially, the column oven temperature 50°C was hold for 5.5 min and then accelerated to 240°C at a rate of 20°C/min, followed by holding at 240°C for 5 min. The acquisition time was 20 min. The injection volume was 1.0 mL with a split ratio of 1:1. The injector temperature was 200°C.

Mass spectroscopy conditions were as follows: Agilent model No. 5977A; Ion source = 230°C; Quad temp. = 150°C; MSD interface line = 240°C; Detector voltage = Gain factor; Ionization mode: Electron impact (EI). The analytes were quantified by gas chromatography electron ionization mass spectrometry (GC-EI-MS) with selective ion mass (SIM). The mass fragment m/z 107 was selected as quantification ion and m/z=87 was selected as qualifier ion for Methyl 2-Bromopropionate. Keep the MS Detector in "Off" mode after 12 min.

HS conditions (Head space): Syringe = 2.5 mL-HS; Sample vol. = 1000µl; Incubation temp. = 80°C; Incubation time = 1200 sec.; Shaking (Agitation) speed = 500 rpm; Shaking (Agitation) on time = 10 sec.; Shaking (Agitation) off time = 10 sec.; Syringe temp. = 100°C; Fill speed = 1500 µl/sec; Pullup delay = 500 msec; Inject to = GC-injection; Inject speed = 1500 µl/sec.; Pre inj. delay = 0 msec.; Post inj. delay = 0 msec.; Syringe flushing = 300 sec.; Analysis time = 1500 sec.

The samples of Tiopronin drug substance obtained from APL Research Centre-II (A division of Aurobindo Pharma Limited). 2-Bromopropionic acid, 2-Bromopropionyl chloride and Sulfuric acid (AR grade) were attained from Merck limited. Methanol and water (HPLC grade) were received from Merck limited.

2.2 Preparation of standard and sample solutions

2-Bromopropionic acid and 2-Bromopropionyl chloride are forms Methyl 2-Bromopropionate with methanol in presence of sulfuric acid. Therefore, in this method 2-Bromopropionic acid and 2-Bromopropionyl chloride are together quantified as Methyl 2-Bromopropionate.

2-Bromopropionic acid is more stable than 2-Bromopropionyl chloride, hence only 2-Bromopropionic acid has been used as standard solution for the determination of 2-Bromopropionic acid, 2-Bromopropionyl chloride development and validation activity.

A derivatizing solution (DS) was prepared by diluting 5 mL concentrated sulfuric acid into a 100 mL clean volumetric flask containing about 50 mL of water, mixed and made upto volume with water. To the head space vial, added 1.0 mL of methanol and 1.0 mL of Derivatizing solution and crimped the vial immediately. This crimped vial was considered as blank. By appropriate weighing, diluting of 2-Bromopropionic acid (Br-PrOH) using methanol solvent to get the concentration about 0.50 µg mL^{-1}, this solution was considered as standard stock solution. To the head space vial, added 1.0 mL of Standard stock solution, 1.0 mL of Derivatizing solution and crimped the vial immediately. This crimped vial was considered as standard. Further, to the HS vial, 0.05 g of Tiopronin sample weighed and transferred, added 1.0 mL of methanol, 1.0 mL of derivatizing solution then crimped the vial immediately. This crimped vial was considered as sample.

Cautions: Handling of sulfuric acid should be done in fumehood. Brominated reference standards are "toxic"; they are commonly referred cancer suspecting reagents. Therefore, in handling of Brominated standards (2-Bromopropionic acid and 2-Bromopropionyl chloride) use protection measurements: wear clothing, personal protective equipment (splash goggles and safety gloves).

3. RESULTS

3.1 Method validation

In the direction to quantify the analytes of 2-Bromopropionic acid and 2-Bromopropionyl chloride in Tiopronin drug substance. The present GC-MS method was validated in terms of selectivity, sensitivity (limits of detection and limits of quantitation), linearity, recovery, precision (system precision and method precision) and range by following the International Conference on Harmonization (ICH) guidelines Q2(R1), step 4 (2005).[10]

3.1.1 Specificity

Selectivity (specificity) is the capability of the analytical method to determine the analyte intensity in presence of Tiopronin drug substance. To explain the selectivity experiment, analyte solutions (2-Bromopropionic acid and 2-Bromopropionyl chloride) were prepared individually and injected into GC-MS to confirm the

retention times and mass fragments. Further, blank solution, control sample or real sample (Tiopronin sample), spiked sample-1 (Tiopronin sample spiked with 2-Bromopropionic acid) and spiked sample-2 (Tiopronin sample spiked with 2-Bromopropionyl chloride) prepared based on method conditions and injected to GC-MS. Based on the chromatograms of individual Br-PrOH, Br-PrCl solutions, blank solution, control sample, spiked sample-1 and spiked sample-2 solutions, it was distinguished that there was no interfering at retention time of Methyl 2-Bromopropionate peak with sample matrix (i.e., no other selected ion mass interference). This demonstrate that the GC-MS method is specific for the quantification of 2-Bromopropionic acid and 2-Bromopropionyl chloride contents as Methyl 2-Bromopropionate in Tiopronin drug substance. A representative chromatogram of (a) blank and (b) standard solution are shown in Figure 1 and a representative chromatogram of (a) control sample (b) spiked sample-1 and (c) spiked sample-2 are shown in Figure 2.

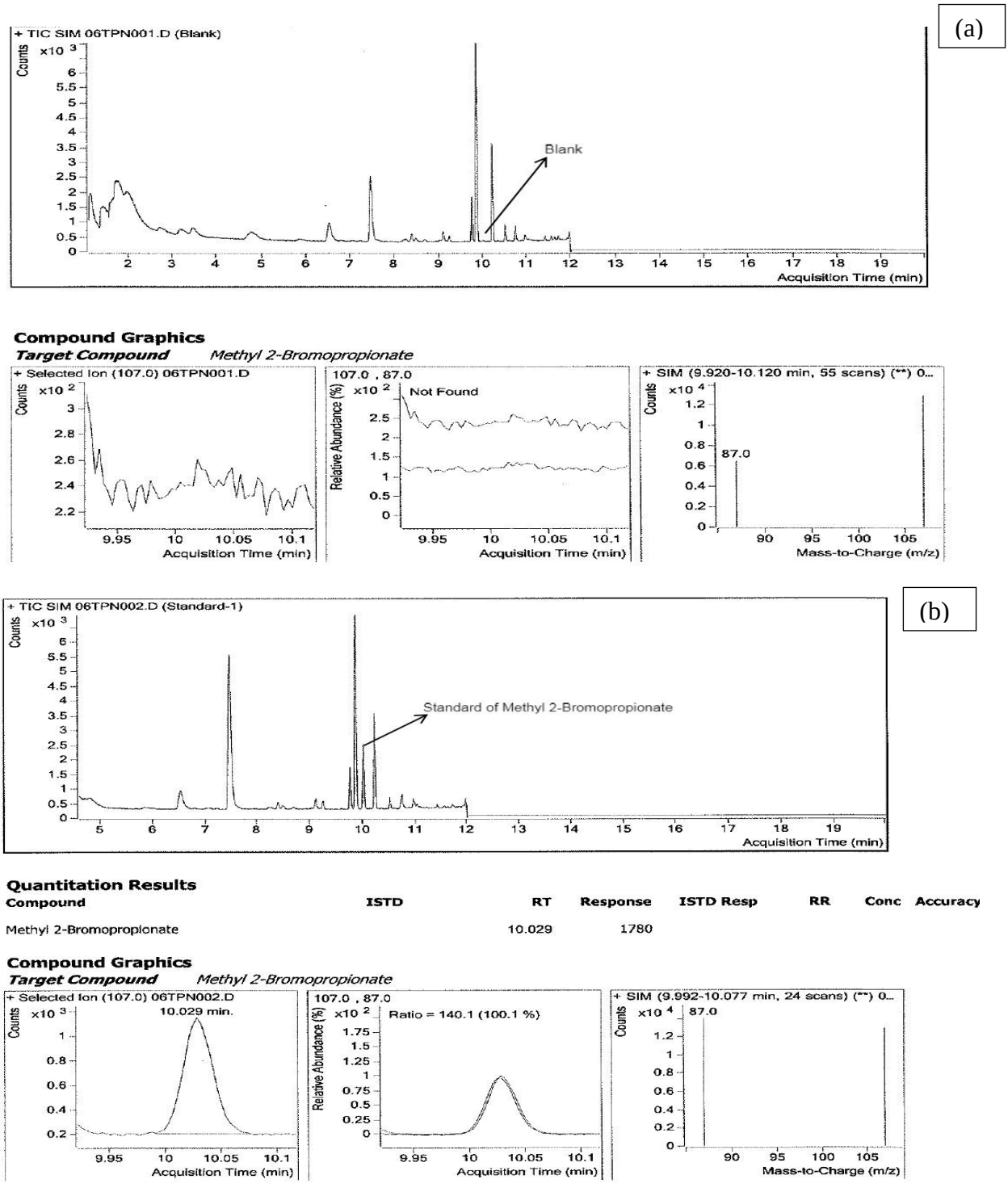
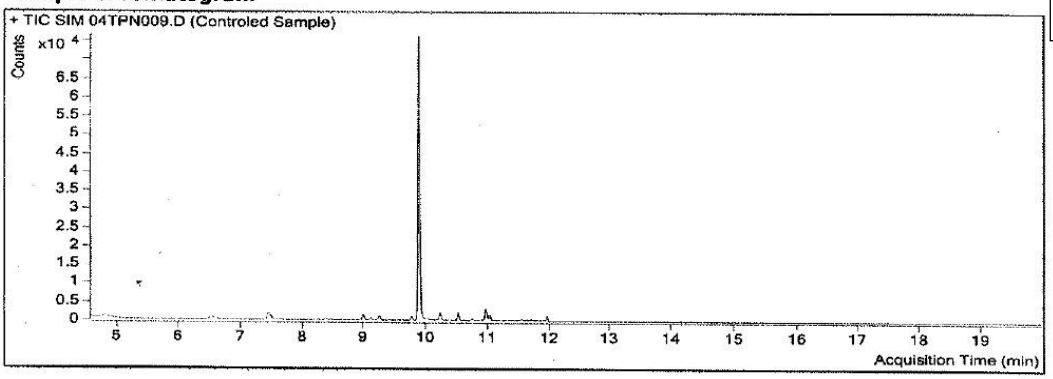


Fig 1. A representative chromatogram of (a) blank and (b) standard solution.

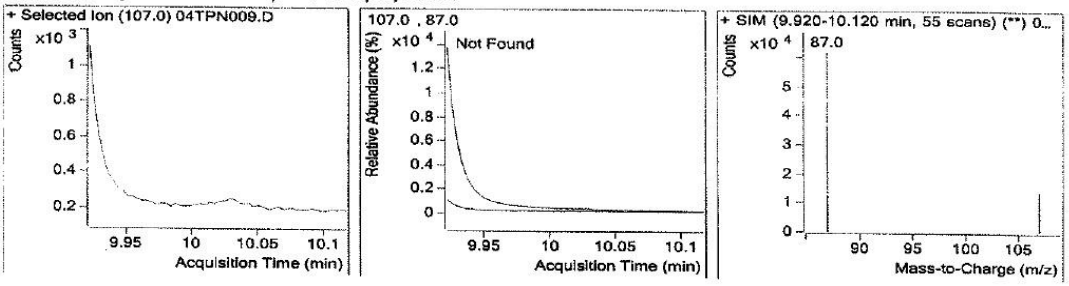
Sample Chromatogram

(a)



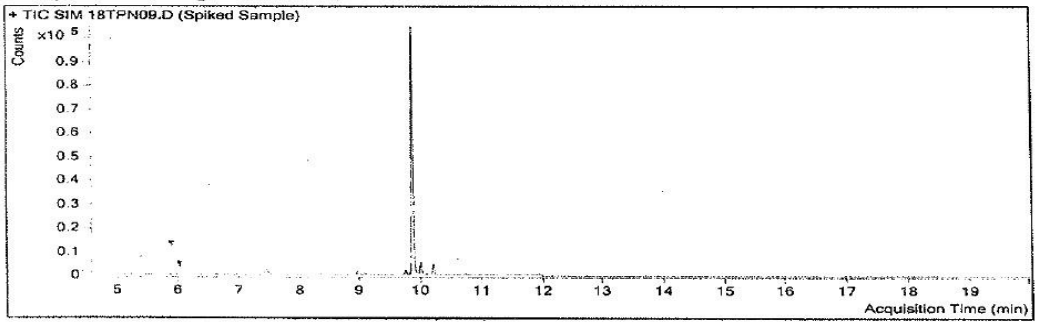
Compound Graphics

Target Compound *Methyl 2-Bromopropionate*



Sample Chromatogram

(b)

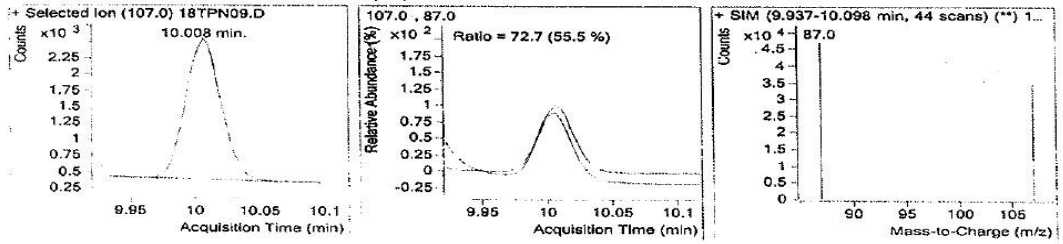


Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
Methyl 2-Bromoopropionate		10.008	4123				

Compound Graphics

Target Compound *Methyl 2-Bromoopropionate*



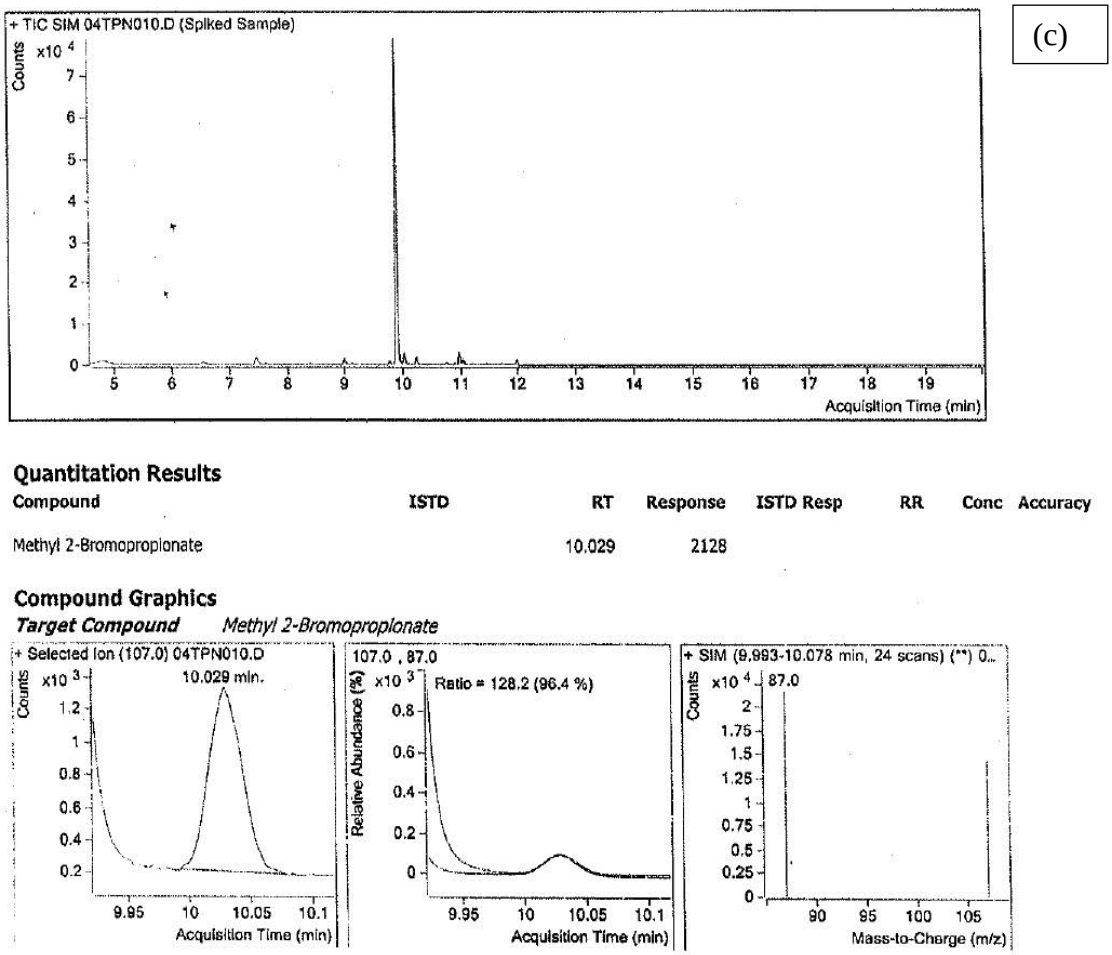


Fig 2: A representative chromatogram of (a)control sample (b)spiked sample-1 & (c)spiked sample-2.

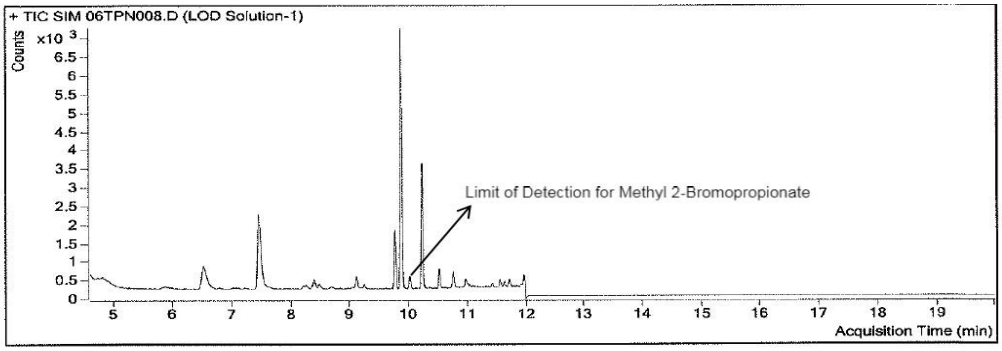
3.1.2 Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) values of 2-Bromopropionic acid as Methyl 2-Bromopropionate was predicted from residual standard deviation and slope of the linearity data demonstrated. The predicted concentrations were confirmed for precision by preparing the solutions, containing 2-Bromopropionic acid, LOD and LOQ solutions were injected six times into GC-MS. The % RSD (relative standard deviation) for six measurements of LOD and LOQ precision were 19.2 and 7.5 respectively. The statistical evaluation of LOD and LOQ precision experiment was detailed in Table 1. A typical representative chromatogram of LOD and LOQ solutions are shown in Figure 3.

Table 1: The statistical evaluation data of Linearity, LOD and LOQ precision experiments.

Statistical parameters	2-Bromopropionic acid (as Methyl 2-Bromopropionate)
Correlation coefficient	0.9992
Intercept	29.555
Residual standard on deviation response	476.448
Slope	86163.946
Concentration range (µg mL^{-1})	0.012 - 0.375
Limit of detection (µg mL^{-1})[a]	0.004
Limit of quantification (µg mL^{-1})[a]	0.012
Precision for Limit of detection (% RSD)	19.2
Precision for Limit of quantification (% RSD)	7.5

a : Precised LOD and LOQ values

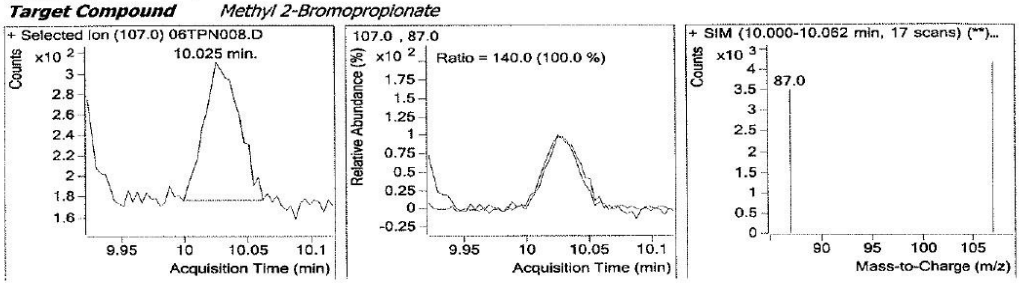


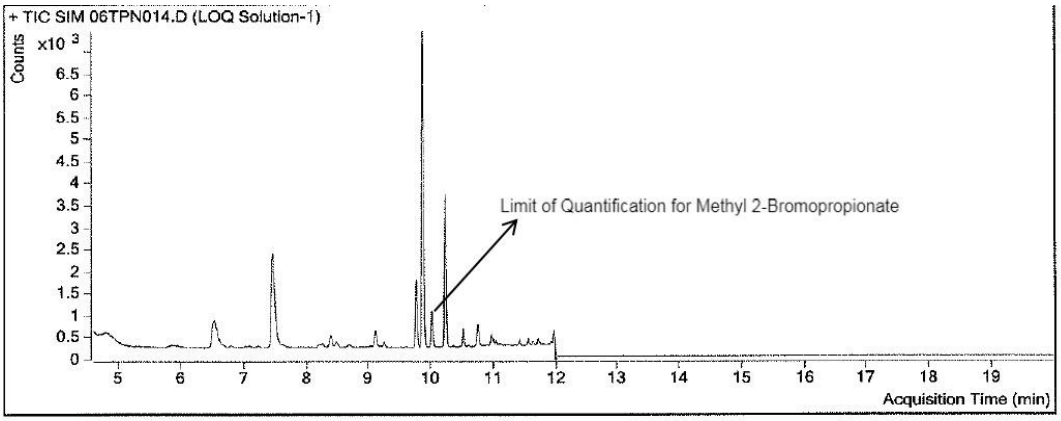
Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
Methyl 2-Bromopropionate		10.025	259				

Compound Graphics

Target Compound *Methyl 2-Bromopropionate*





Quantitation Results

Compound	ISTD	RT	Response	ISTD Resp	RR	Conc	Accuracy
Methyl 2-Bromopropionate		10.029	656				

Compound Graphics

Target Compound *Methyl 2-Bromopropionate*

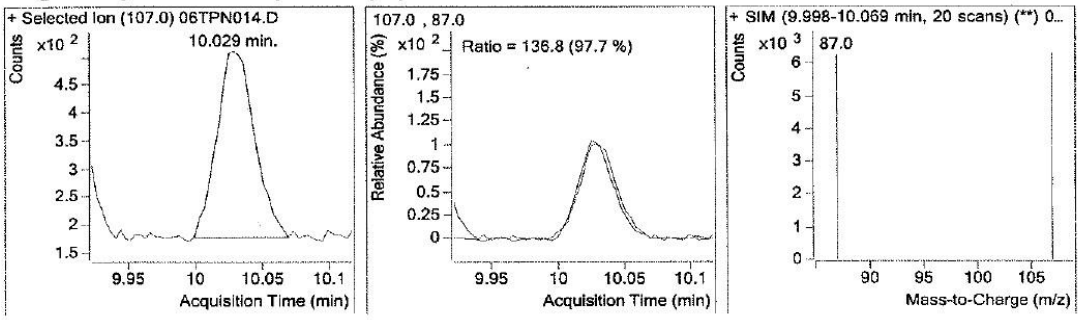


Fig 3: A typical representative chromatogram of LOD and LOQ solution.

3.1.3 Linearity

Linearity experiment was practically carried out by measuring the analyte response of seven different aliquots of 2-Bromopropionic acid (as Methyl 2-Bromopropionate) were prepared across the range of 0.012 µg mL^{-1} to 0.375 µg mL^{-1}. The seven aliquots of linearity solutions 0.012, 0.075, 0.125, 0.200, 0.250, 0.300 and 0.375 µg mL^{-1} were prepared and injected each solution single time into GCMS. The obtained experimental values were applied for statistical evaluation using a linear-regression model. The obtained correlation co-efficient value for linearity experiment was 0.9992. The statistical parameters such as slope, intercept, residual standard on deviation and correlation coefficient results were calculated. Linearity results are detailed in Table 1.

3.1.4 Accuracy

The recovery (accuracy) testing was executed by spiking known quantities of 2-Bromopropionic acid at LOQ level, 50%, 100% and 150% levels (with respect to 10 µg g^{-1} level) to Tiopronin drug substance. As a part of recovery experiment, Tiopronin sample [as such sample (or) control sample] HS vials prepared triplicate without spiking 2-Bromopropionic acid and injected into GC-MS. After that, Tiopronin sample spiked with 2-Bromopropionic acid at four different concentration levels [LOQ level = 0.45 µg g^{-1}; 50% level = 5 µg g^{-1}; 100% level = 10 µg g^{-1} and 150% level = 15 µg g^{-1}] and each level prepared into triplicate and injected to GC-MS. By analyzing all the accuracy samples and the obtained accuracy values are applied for percentage recovery calculations. The mean % accuracy results of four levels (LOQ, 50%, 100% and 150% levels) of twelve measurements was 99.4%. The experimental recovery results are detailed in Table 2.

Table 2: The validated accuracy results for 2-Bromopropionic acid.

Identification	2-Bromopropionic acid (as Methyl 2-Bromopropionate)			
	LOQ Level	50% Level	100% Level	150% Level
*Amount added (µg g^{-1})	0.46	5.07	10.17	15.15
*Amout found (µg g^{-1})	0.48	4.97	10.14	14.69
Recovery (%)	102.9	98.0	99.6	97.0
* % RSD	14.3	3.1	4.3	2.4
Average recovery for LOQ, 50%, 100% and 150% levels	99.4%			

* Average of 3 replicates

3.1.5 Precision

The precision experiment (system precision and method precision) of the method was studied by taking 2-Bromopropionic acid standard solution (0.25 µg mL^{-1}) and injected in six replicates for system precision. Separately, six preparations of Tiopronin sample spiked with 2-Bromopropionic acid at about 10 µg g^{-1} level and injected into GC-MS for method precision experiment. In addition to that, the same method precision experiment has also been demonstrated separately by spiking with 2-Bromopropionyl chloride at about 10 µg g^{-1} level and injected into GC-MS. The precision values are subjected to statistical calculation (mean, standard deviation and % relative standard deviation). The % RSD results obtained for system precision was 6.0% (Acceptance: % RSD to be less than 10.0%) and the obtained % RSD results for method precision was 4.4 (2-Bromopropionic acid) and 3.4 (2-Bromopropionyl chloride). The concluded values of system precision and method precision of the test method showed that this method is sufficiently precise. The precision values are presented in Table 3.

Table 3: The precision experimental results.

Repeatability (System precision) Area		
	2-Bromopropionic acid (as Methyl 2-Bromopropionate)	
1	19300	
2	22346	
3	20775	
4	19383	
5	20150	
6	21697	
Mean	20609	
SD	1237	
% RSD	6.0	
Reproducibility (Method precision) (µg g^{-1})		
	2-Bromopropionic acid (as Methyl 2-Bromopropionate)	2-Bromopropionyl chloride (as Methyl 2-Bromopropionate)
1	10.4	10.3

2	9.8	10.2
3	10.4	10.3
4	10.3	11.0
5	10.3	10.9
6	9.3	10.3
Mean	10.1	10.5
SD	0.44	0.35
% RSD	4.4	3.4

4. DISCUSSION

4.1 Method development and optimization

The aim of the current effort is, to develop a fabulous GC-MS method for the quantification of 2-Bromopropionic acid (Br-PrOH) and 2-Bromopropionyl chloride (Br-PrCl) in Tiopronin drug substance. Development activity was started as per Tiopronin, Br-PrOH and Br-PrCl solubility and miscibility studies. Due to poor response of Br-PrOH and Br-PrCl in UV detection, there is no possibility by HPLC. Since, Br-PrOH and Br-PrCl are volatile in nature, there is a option to develop a method by using gas chromatograph (GC) equipped with flame ionization detector (FID). Preliminary experiments were carried out based on the retention times of organic acids, that were discussed in the J&W GC column application guide. By using DB-FFAP column (30 m x 0.32 mm, 0.5 µm), consists of nitro terephthalic acid modified polyethylene glycol as stationary phase, that provides very inert and designed mainly for organic acids and free fatty acids. Elution of analytes were investigated using carrier gas as helium, with the column flow 1.5 mL/min. Initially, the column oven temperature of 50°C was hold for 5.5 min and then accelerated to 240°C with the rate of 20°C/min, followed by holding at 240°C for 5 min. The injection volume was 1.0 mL with a split ratio of 1:1. From this experimental trial, Br-PrOH and Br-PrCl were not clearly separated from sample matrix peak and broad peak shapes were observed. For clear separation and better peak shapes, again trials were performed by changing the column, i.e., ZB-624, (30 m x 0.32 mm, 1.8 µm) capillary column coated with 6% cyanopropylphenyl and 94% dimethylpolysiloxane material as a stationary phase by retaining same chromatographic conditions. From this experimental trial, Br-PrOH and Br-PrCl were separated sample matrix peak with low response and recovery result was about 150%. Due to the low response of these impurities in GC with FID, we have chosen a gas chromatography electron ionization mass spectrometry (GC-EI-MS) technique in selective ion monitoring (SIM) mode by following the derivatization procedures to meet the genotoxic limit (based on TTC approach, the limit is not more than 10 µg g^{-1}). Elution of Br-PrOH and Br-PrCl were investigated using ZB-624, (30 m x 0.32 mm, 1.8 µm) capillary column by using the same chromatographic conditions and employing boran trifluoride in methanol solvent as derivatizing reagent. The reaction mechanism involved in the derivatization process, both analytes (2-Bromopropionic acid and 2-Bromopropionyl chloride) are converted into Methyl 2-Bromopropionate. In this experimental trial, sample matrix interference was observed and recovery results was about 40%. After that, to improve recovery results, again trial was performed by changing the derivatizing reagent i.e., sulfuric acid in methanol and remaining chromatographic conditions were kept as same. In this derivatization process, 2-Bromopropionic acid and 2-Bromopropionyl chloride analytes are converted into Methyl 2-Bromopropionate in presence of sulfuric acid and methanol. The recovery results are found satisfactory.

Finally, satisfactory recovery results were achieved, on chromatographic conditions which had been mentioned in instrumentation and applied for validation activity to evaluate its performance characteristics.

5. CONCLUSION

In conclusion, the author reveals a well resolved analytical method for the quantification of 2-Bromopropionic acid (Br-PrOH) and 2-Bromopropionyl chloride (Br-PrCl) in Tiopronin drug substance by GC-EI-MS with SIM mode at TTC levels. The reaction mechanism involved in the derivatization process, both Br-PrOH and Br-PrCl analytes are converted into Methyl 2-Bromopropionate in presence of sulfuric acid and methanol. Method validation data confirmed that the method is dynamic, sensitive, specific, precise, linear, accurate, user friendly and cost-effective for the estimation of Br-PrOH and Br-PrCl, quantified as Methyl 2-Bromopropionate in Tiopronin drug substance.

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