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A RAPID SENSITIVE AND ACCURATE LCMS METHOD FOR THE DETERMINATION OF MUTAGENIC IMPURITIES IN MOXIFLOXACIN HCL

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ABSTRACT

A quantitative method was developed and then validated for two genotoxic impurities (GTS-STG-1A and GTS/STG-1B) in moxifloxacin hydrochloride API samples using the LC–MS approach. The analytes (GTS-STG-1A and GTS/STG-1B) were separated on XDB- column [C8; Eclipse; 5 µm particle dimension; 150 mm length; and ID of 4.6 mm] using a blend of solvent systems 'A' (10 mM strength ammonium formate) and 'B' (pure acetonitrile) in a gradient pattern elution. The GTS-STG-1A and GTS/STG-1B were detected by positive mode electrospray ionization. The transitions at m/z 332.1–286.2 and 344.2–298.4 were used for GTS-STG-1A and GTS/STG-1B quantitative analysis. Better linearity for the concentration ranges of 0.3863 ppm to 5.7938 ppm for GTS-STG-1A and 0.3786 ppm to 5.6790 ppm for GTS/STG-1B was obtained. The accuracies (94.6% to 99.35 for GTS-STG-1A and 83.25% to 105.9% for GTS-STG-1B) and precisions (1.40% for GTS-STG-1A and 4.30% for GTS-STG-1B) fell within the allowable threshold. The GTS-IA and GTS-IB demonstrated adequate stability under-examined conditions. By keeping track of the GTS-STG-1A and GTS/STG-1B content in the moxifloxacin hydrochloride API during the experiment, the usefulness of the LC-MS technique was shown.

Keywords: Antibiotic, Genotoxic Impurities, Mass Spectrometer, Validation, Quality Check.
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INTRODUCTION

The broad-spectrum antibiotic, moxifloxacin hydrochloride (MXH), stops bacterial genetic material (DNA) replication by blocking the DNA gyrase enzyme, type 2 topoisomerase enzyme, and topoisomerase 4 enzyme.[1] This blocks bacterial cells from replicating. MXH is a drug used to manage conjunctivitis, skin infections, pre & post-operative conditions as well as respiratory infections including pneumonia & bronchitis.[2-4] MXH is available in tablet form, ophthalmic solution form, and infusion bag form.[5-7] Figure-1 depicts the MXH chemical manufacturing pathway.[8] One of the intermediates in the chemical route of making MXH is gatiester. Gatiester is chemically described as "1-Cyclopropyl-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinoline carboxylic acid ethyl ester". The "2,4,5, trifluoro-3-methoxy benzoyl chloride" and "ethyl 3-(N,N-dimethylamino)acrylate" are the precursors of gatiester synthesis. During gatiester formation from precursors, Ethyl-3-dimethylamino)-2-(2,4,5-trifluoro-3-methoxybenzoyl) acrylate (GTS-STG-1A) and Ethyl-3-(cyclopropylamino)-2-(2,4,5-trifluoro-3-methoxybenzoyl) acrylate (GTS/STG-1B). The GTS-STG-1A and GTS/STG-1B are regarded as genotoxic impurities relying on the Vega software evaluations. Even at incredibly low quantities, genotoxic impurities (GNTIs) can engage with human DNA and result in cancerous mutations.[9] Risks related to medication safety and purity can be avoided by spotting GNTIs early in the drug manufacturing process and reducing GNTIs at tolerable concentrations in the API molecule or pharmaceutical final product.[10,11] As a result, a novel and effective approach for the detection including measurement of trace GNTI levels has to be created. Highly sensitive and focused analytical techniques that are appropriate for the target sample matrices are needed to detect GNTIs at trace levels. The GC-MS, UPLC-MS, and LC-MS technologies were applied to quantify GNTIs at trace levels due to their great sensitivity.[12-21]

Djurdjevic *et al*., (RP-HPLC), Vankalapati *et al.*, (RP-HPLC), Cai *et al.,* (HPLC), and Li *et al.,* (LC-MS/MS) described analytical methods for MXH-related impurities and MXH metabolic intermediate.[22-25]

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The GNTIs, GTS-STG-1A, and GTS/STG-1B were not addressed in any of the procedures described.[22-25] As a necessary consequence, we were able to establish a quick, accurate, and sensitive liquid chromatographic approach for the detection of GTS-STG-1A and GTS/STG-1B in MXH API forms.

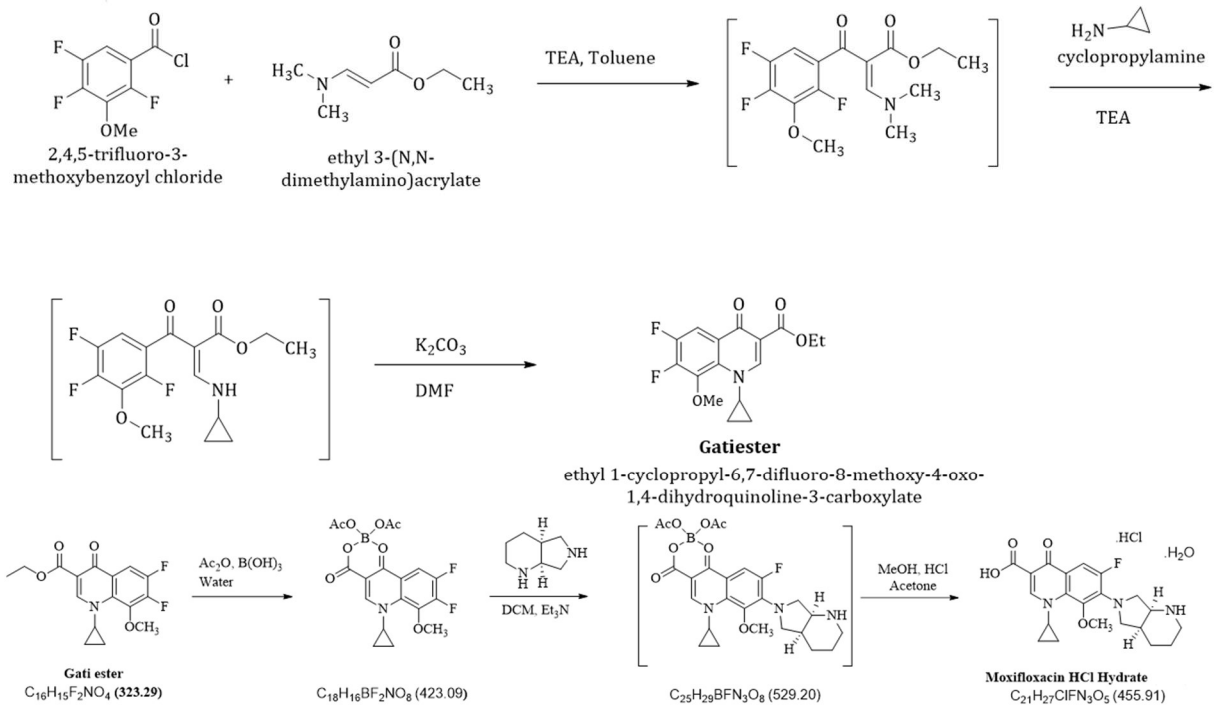

Fig.-1: Gatiester and MXH Chemical Manufacturing Pathways

EXPERIMENTAL

LC-MS Equipment

Analysis of GTS-STG-1A and GTS/STG-1B were made using Thermo Scientifics make "Electrospray ionization triple quadruple mass spectrometer".

Chromatography Column

Separation of GTS-STG-1A and GTS/STG-1B was made using XDB- column [C8; Eclipse; 5 µm particle dimension; 150 mm length; and ID of 4.6 mm].

Reference Standards and Samples

Reference standards of GTS-STG-1A and GTS/STG-1B and samples of MXH API forms were provided as gifts from "Aragen Life Sciences", India.

Chemicals

"Sigma-Aldrich" make reagent graded ammonium formate and "Merck" make HPLC graded acetonitrile and Milli Q water was used in GTS-STG-1A and GTS/STG-1B simultaneous analysis.

LC-MS Conditions

The solvent systems 'A' (10 mM strength ammonium formate) and 'B' (pure acetonitrile) were combined to perform a gradient elution (Table-1) analysis of GTS-STG-1A and GTS/STG-1B. The temperatures were 45 ºC (at the column) and 5 ºC (at the sample port). We employed a sample loop and injected 20 µL for analysis. The operating mobile phase flow was 1.0 mL/min. The electron spray ionization was used in positive mode to run the mass spectrometer apparatus. Table-1 displays the distinct SRM transitions as well as other experimental factors. Software called Thermo Scientific™ Xcalibur™ was deployed to process the data. An 8:2 (*v/v*) mixture of pure acetonitrile and water was the diluent for sample preparations.

Table-1: Gradient Program and MS Conditions

Gradient program		
Time	Solvent system 'A' (%)	Solvent system 'B' (%)
0.0	60	40
20.0	50	50
20.1	60	40
25.0	60	40
MS conditions		
Parameters	GTS-STG-1A	GTS-STG-1B
Precursor ion (m/z)	332.1	344.2
Product ion (m/z)	286.2	298.4
Collision Energy(V)	12	15
Positive Ion (V)	3500	3500
Negative Ion (V)	2500	2500
Vaporizer temperature (°C)	350	350
Ion transfer tube temperature(°C)	380	380

Standard Solutions

The stock GTS-STG-1A and GTS/STG-1B solution of 250 ppm concentration each were prepared to deploy diluent. The working GTS-STG-1A and GTS/STG-1B solution of 3.75 ppm concentration each were prepared thru proper stock GTS-STG-1A and GTS/STG-1B solution dilution deploying diluent.

Calibration Curves of GTS-STG-1A and GTS/STG-1B

Six sets of calibrators (0.3863, 1.9313, 3.0900, 3.8625, 4.6350, 5.7938 ppm of GTS-STG-1A and 0.3876, 1.8930, 3.0288, 3.7860, 4.5432, 5.6790 ppm of GTS-STG-1B) were made from stock GTS-STG-1A (250 ppm) and GTS/STG-1B (250 ppm) solution and examined by proposed LC-MS method. The regression coefficients of GTS-STG-1A and GTS/STG-1B were computed in order to explore GTS-STG-1A and GTS/STG-1B calibration curves.

Analysis of GTS-STG-1A and GTS/STG-1B in MXH API

Three MXH API samples were chosen to estimate the total GTS-STG-1A and GTS/STG-1B content using the suggested LC-MS technique. A standard flask (50 mL) was loaded with 25 mg of MXH API. A 25 mL of diluent was then added, mixed, and ultrasonic bathed for 5 min to completely dissolve the MXH API. Following that, the appropriate amounts of diluent were placed into the same flask. The proposed LC-MS technique was applied to investigate the produced MXH API sample for total GTS-STG-1A and GTS/STG-1B content.

RESULTS AND DISCUSSION

LC-MS Technique Optimization

To optimize the mobile phase for GTS-STG-1A and GTS/STG-1B analysis, two combinations were investigated. In the first, solvent systems "A" (10 mM strength ammonium formate) and "B" (pure acetonitrile) are used. The second combination is made up of the solvent systems "A" (0.5% formic acid) and "B" (pure acetonitrile). The response of GTS-STG-1A and GTS/STG-1B in the formic acid mobile phase was found less when compared to the ammonium formate system. Three columns [Atlantis column T3,150 mm × 4.6 mm, 5 µm; XDB column C8, Eclipse 250 mm × 4.6 mm, 5 µm; and XDB column C8, Eclipse 150 mm × 4.6 mm, 5 µm] were investigated. Atlantis column T3 revealed a poor peak shape for GTS-STG-1B. Peak shape for GTS-STG-1B was found good in XDB column C8, Eclipse 250 mm × 4.6mm, 5 µm, but responses of GTS-STG-1A and GTS/STG-1B were poor. Better response for GTS-STG-1A and GTS/STG-1B was obtained with XDB column C8, Eclipse 150 mm × 4.6mm, 5 µm.

Three gradient runs (60, 45, and 25 min) using XDB column C8, Eclipse 150 mm × 4.6mm, 5 µm and pure acetonitrile as modifier (solvent system 'B') and 10 mM strength ammonium formate (solvent system 'A') were tested. Better response with reasonable time for GTS-STG-1A and GTS/STG-1B analysis was found

with gradient program: Gradient program: 0 min 40% 'B'; 20 min 50% 'B'; 20.1 min 40% 'B'; and 25 min 40% 'B'. Tried two different flow rates: 0.8 mL/min and 1.0 mL/min. With a 1.0 mL/min flow rate, the peaks of GTS-STG-1A and GTS/STG-1B were resolved better with retention times of 6.76 min and 14.69 min, respectively. First positive and then negative forms of electrospray ionization were tested, and the positive mode was preferred since it generated high levels of GTS-STG-1A and GTS/STG-1B intensity. Obtained the maximum sensitive transitions at *m/z* 332.1–286.2 for GTS-STG-1A and 344.2–298.4 for GTS/STG-1B.

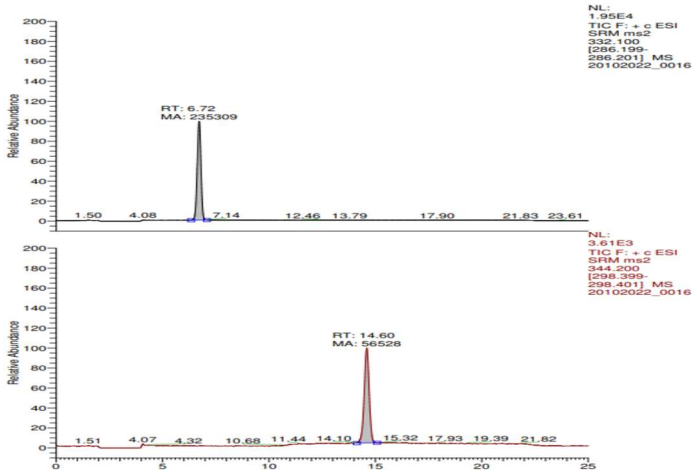

Fig.-2: LC-MS Chromatogram for GTS-STG-1A and GTS/STG-1B with Optimized Settings

Validation

The linear range, LODs, LOQs, analyte recovery, accuracy, robustness, and precision were all taken into account when validating the method as per the criteria of ICH.[26]

Specificity

The working GTS-STG-1A (3.75 ppm) and GTS/STG-1B (3.75 ppm) solution, diluent blank (8:2 (v/v) mixture of pure acetonitrile and water), and spiked MXH API solution (3.75 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B) were analyzed individually thru suggested LC-MS technique and assessed for interference thru comparison of respective LC-MS chromatograms (Fig.-3 to 5). It was demonstrated that neither the diluent nor the MXH API significantly interfered with GTS-STG-1A or GTS/STG-1B peaks.

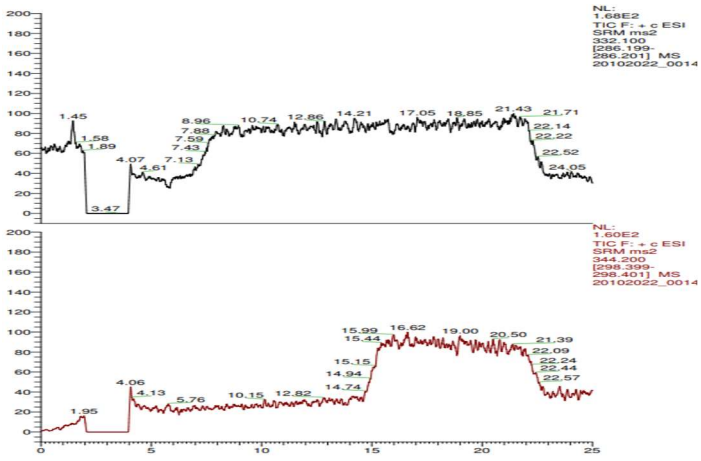

Fig.-3: Diluent Blank (8:2 (v/v) Mixture of Pure Acetonitrile and Water) LC-MS Chromatogram

Precision

Six replicates of each of the spiked MXH API samples (3.75 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B) were evaluated to appraise LC-MS technique precision. The SD and RSD of the repetitive GTS-STG-1A and GTS/STG-1B content measurements were chosen to represent LC-MS technique precision (Table-2). Precision associate measurements were revealed to be under 15% (tolerable measures).

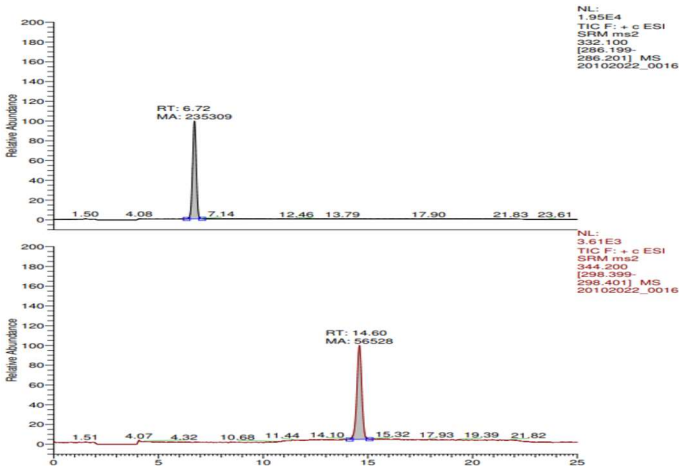

Fig.-4: Working GTS-STG-1A (3.75 ppm) and GTS/STG-1B (3.75 ppm) Solution LC-MS Chromatogram

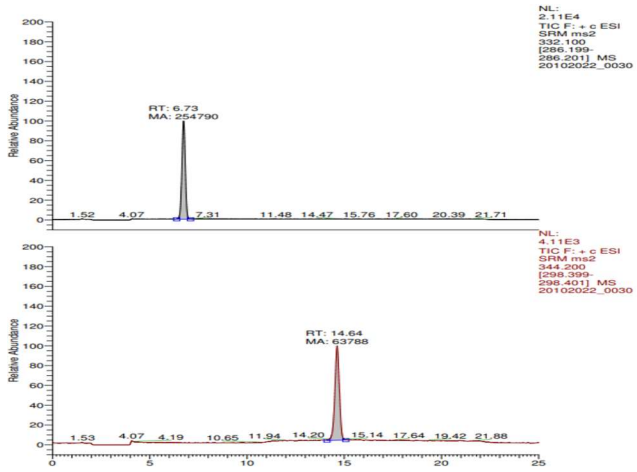

Fig.-5: Spiked MXH API Solution (3.75 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B) LC-MS Chromatogram

Table-2: Appraisal of LC-MS Technique Precision

Weight of the MXH (mg)	GTS-STG-1A		GTS/STG-1B	
	Area	Content (ppm)	Area	Content (ppm)
25.48	355027	3.39	95310	3.27
25.44	358125	3.42	101024	3.48
25.15	362066	3.50	107268	3.73
25.40	366307	3.50	102697	3.54
25.30	361057	3.47	103952	3.60
25.40	357123	3.42	103323	3.56
Statistical evaluation →	Avg.	3.45	Avg.	3.53
	STDEV	0.0484	STDEV	0.1518
	%RSD	1.40	%RSD	4.30

Sensitivity

Quantification limit (QL) and detection limit (DL) were gauged to assess LC-MS technique sensitivity. The lowest detectable levels (DLs) of GTS-STG-1A and GTS/STG-1B were estimated by applying a signal response-to-noise (S/N) proportion of three. The lowest measurable quantity (QLs) for GTS-STG-1A and GTS/STG-1B was evaluated by applying a signal response-to-noise (S/N) proportion of ten. The DLs for GTS-STG-1A and GTS/STG-1B were 0.1159 ppm and 0.1136 ppm, respectively while QLs were 0.3863 ppm and 0.3786 ppm for GTS-STG-1A and GTS/STG-1B, respectively. Three replicates of standard GTS-STG-1A (0.1159 ppm) & GTS/STG-1B (0.1136 ppm) solution and six replicates of standard GTS-STG-

1A (0.3863 ppm) & GTS/STG-1B (0.3786 ppm) solution were evaluated to appraise LC-MS technique precision at DL level and QL level concentrations. The SD and RSD of the repetitive GTS-STG-1A and GTS/STG-1B peak response area measurements were made (Table-3). Precision associate measurements at DL level and QL level concentrations were revealed to be under 20% (tolerable measures).

Table-3: Appraisal of LC-MS Technique Precision at DL Level and QL Level Concentrations

Injections	GTS-STG-1A		GTS/STG-1B	
	Content (ppm)	Area	Content (ppm)	Area
Quantification level precision				
1	0.3863	24459	0.3786	4919
2	0.3863	24505	0.3786	3856
3	0.3863	25937	0.3786	4240
4	0.3863	24739	0.3786	4179
5	0.3863	25276	0.3786	4666
6	0.3863	25437	0.3786	5437
Statistical evaluation →	Avg.	25059	Avg.	4550
	STDEV	588.2470	STDEV	574.7628
	%RSD	2.35	%RSD	12.63
Detection level precision				
1	0.1159	9022	0.1136	1762
2	0.1159	8334	0.1136	1689
3	0.1159	8421	0.1136	1233
Statistical evaluation →	Avg.	8592	Avg.	1561
	STDEV	374.6363	STDEV	286.6781
	%RSD	4.36	%RSD	18.36

Linearity

The initial concentrations of GTS-STG-1A and GTS/STG-1B calibration curves were its accepted QLs. The least squares regression strategy was implemented to create calibration lines for the concentration ranges of 0.38 ppm to 5.80 ppm for GTS-STG-1A and 0.38 ppm to 5.70 ppm for GTS/STG-1B. The GTS-STG-1A and GTS/STG-1B were both noticed to have correlation coefficient values (R^2) of 0.9999.

Regression equation for GTS-STG-1A: $y = 692208x - 5557$
Regression equation for GTS/STG-1B: $y = 204095x + 1342.1$

Accuracy

Three replicates of each of the spiked MXH API samples at QL level (0.38 ppm of GTS-STG-1A and 0.37 ppm of GTS/STG-1B), at 50% level (1.91 ppm of GTS-STG-1A and 1.88 ppm of GTS/STG-1B), at 100% level (3.83 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B) and at 150% level (5.73 ppm of GTS-STG-1A and 5.62 ppm of GTS/STG-1B) were evaluated to appraise LC-MS technique accuracy and recovery properties. The recovery of GTS-STG-1A and GTS/STG-1B content measurements were chosen to represent LC-MS technique accuracy (Table - 4). For GTS/STG-1B and GTS-STG-1A, the accuracy valuations vary from 83.25% to 105.9% and 94.6% to 99.35, respectively. These counts were deemed suitable.

Table-4: Appraisal of LC-MS Technique Accuracy and Recovery

GTS-STG-1A			GTS/STG-1B		
Level (%)	Added (ppm)	% Recovery[a]	Level (%)	Added (ppm)	% Recovery[a]
QL	0.38	94.60	QL	0.37	91.02
50	1.91	98.5	50	1.88	105.9
100	3.83	99.3	100	3.75	96.3
150	5.73	94.9	150	5.62	83.2

a-mean for recovered values (n=3)

Solution Stability

The spiked MXH API samples (3.75 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B) were used to appraise GTS-STG-1A and 3.75 ppm of GTS/STG-1B stabilities in solution. Two spiked MXH API samples were preserved, one at the bench top and the other in the refrigerator (2–8°C). Analyzed the preserved (Room temperature & 2-8°C) spiked MXH API samples at Initial, 7 hr, and 24-hour time intervals. The percentage comparative difference (Table - 5) of GTS-STG-1A and GTS-STG-1B response area from initial and after each interval was within ± 20% (tolerable measures). Thus, it was established that solutions of GTS-STG-1A and GTS-STG-1B were sustainable for up to 24 hr at benchtop temperatures as well as between 2 ºC and 8 ºC.

Table-5: GTS-STG-1A and GTS-STG-1AB Stability at Bench Top and at 2-8°C

Time	Weight of the MXH (mg)	GTS-STG-1A		GTS/STG-1B	
		Area	Content (ppm)	Area	Content (ppm)
Initial	25.34	360411	3.51	96812	3.30
7 hr (bench top)	25.34	358420	3.49	98434	3.35
%Relative difference			0.55	-	-1.60
7 hr (2-8 °C)	25.34	354133	3.45	99671	3.40
%Relative difference			1.74	-	-2.90
24 hr (bench top)	25.34	351797	3.42	101713	3.47
%Relative difference			2.39	-	-5.06
24 hr (2-8 °C)	25.34	356355	3.47	108360	3.69
%Relative difference			1.13	-	-11.93

Robustness

The robustness of the LC-MS technique was assessed with spiked MXH API samples (3.75 ppm of GTS-STG-1A and 3.75 ppm of GTS/STG-1B). The investigated conditions were low rate of flow (0.8 mL/min), high rate of flow (1.2 mL/min), column low temperature (43 °C), column high temperature (47 °C), Sheath gas high flow (53), and Sheath gas low flow (43). The percentage comparative difference (Table-6) of GTS-STG-1A and GTS-STG-1B response area from the optimized LC-MS technique set and after each LC-MS technique set variation was gauged. The values have not exceeded the variation of 20% (tolerable measures).

Table-6: Appraisal of LC-MS Technique Robustness

Condition	GTS-STG-1A Content (ppm)	GTS-STG-1B Content (ppm)
As such	3.81	4.06
Low flow	3.48	3.89
%Relative difference	8.80	4.31
High flow	3.87	3.71
%Relative difference	-1.43	8.66
Low temperature	3.95	3.66
%Relative difference	-3.68	10.02
High temperature	3.91	3.66
%Relative difference	-2.47	10.02
Low Gas	3.83	3.65
%Relative difference	-0.58	10.08
High Gas	3.73	3.52
%Relative difference	2.16	13.42

Application to the Batch Analysis

The chosen GNTIs (GTS-STG-1A and GTS-STG-1B) were assessed in three batches of MXH API samples, which were then used to evaluate the LC-MS technique's applicability. We noticed the complete absence of GTS-STG-1B and 0.01 ppm content of S-STG-1A in MF200122, MF200222and MF200322 batches of MXH API samples. These samples passed the quality control check as content levels of GTS-STG-1B and S-STG-1A were within their specification (3.75 ppm) content limit.

CONCLUSION

A quantitative methodology was developed for two GNTIs (GTS-IA and GTS-IB) in MHX API samples using the LC–MS approach. Due to its 0.11 ppm detection limit, the LC-MS technique offered greater sensitivity for GTS-IA and GTS-IB analysis. Decent reproducibility, excellent accuracy, negligible diluent influence, and acceptable robustness were exploited to support the LC-MS technique's reliability for GTS-IA and GTS-IB analysis in MXH API samples.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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