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NOVAL METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF THREE POTENTIAL GENOTOXIC IMPURITIES IN DARUNAVIR PROPYLENE GLYCOLATE DRUG SUBSTANCE BY LC-MS/MS TECHNIQUE

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Keywords:

Liquid Chromatography-Mass Spectrometry (LC-MS/MS), Potential Genotoxic Impurities (PGI), active pharmaceutical ingredient (API) and Darunavir propylene glycolate

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ABSTRACT: A Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method was used for quantification of potential genotoxic impurities (PGI) in the Darunavir propylene glycolate drug substance (API). Chromatographic separation was achieved using a column UPLC BEH C18, 1.7 µm (100×2.1mm), with a Mobile-phase-A was ammonium bi carbonate and Mobile-phase-B was acetonitrile in gradient elution mode at a flow rate of 0.3 ml/min and injection volume is 2.0 µL. Quantification of impurities was carried out using triple quadrupole mass detection with electrospray ionization in multiple reaction monitoring mode. The method was fully validated with good linearity over the concentration range of 0.23 to 1.69 µg/g of the Darunavir propylene glycolate test concentration for all Genotoxic impurities. The correlation coefficient obtained in each case was 0.999. The recoveries were found satisfactory over the range between 98.1 to 101.4% for all selected impurities. A novel selective, highly sensitive and hyphenated analytical method using LC-MS/MS coupled with negative electrospray ionization has been developed for quantification of genotoxic impurities like Nitro benzene sulfonic acid and Methyl-4-amino benzene sulfonate at in Darunavir propylene glycolate API.

INTRODUCTION: Darunavir propylene glycolate [(1S, 2R)-3-(((4-aminophenyl) sulfonyl) (2 - methylpropyl) amino) – 2 – hydroxyl - d1-(phenylmethyl) propyl] carbamic acid (3R, 3aS, 6aR)-hexahydrofuro (2,3-b) furan-3-yl ester, 1,2-propanediol chemical structure shown in **Fig. 1**. Darunavir propylene glycolate is a new HIV peptide protease inhibitor (PI). It acts on HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments.

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Reg. the genotoxic and carcinogenic impurities in API, a draft of guidelines also outlined by US FDA. The consists of the different various routes to mitigate the potential lifetime cancer risk in patients with exposure to genotoxic impurities. Based on the current regulatory guidance for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5 µg/day daily intake of individual impurity [1-3].

The Darunavir propylene glycolate drug substance has potential genotoxic impurities, namely 4-Nitro benzene sulfonic acid, 4-Nitro benzene sulfonil chloride, Methyl-4-amino benzene sulfonate. In this present study we developed a LC-MS/MS method to determine the 4-Nitro benzene sulfonic acid, 4-Nitro benzene sulfonil chloride and Methyl-4-amino benzene sulfonate a potential genotoxic

impurity in Darunavir propylene glycolate drug. 4-Nitro benzene sulfonyl chloride is converted into 4-Nitro benzene sulfonic acid hence, by this procedure any amount of 4-Nitro benzene sulfonyl chloride and 4-Nitro benzene sulfonic acid present in Darunavir propylene glycolate quantified as 4-Nitro benzene sulfonic acid [4]. Refer below for the schematic diagram for Darunavir propylene glycolate shown in **Fig. 2** and all impurities shown in **Table 1.**

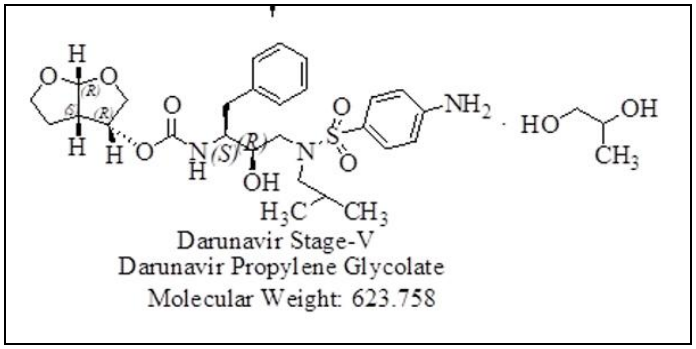


FIG. 1: DARUNAVIR PROPYLENE GLYCOLATE CHEMICAL STRUCTURE

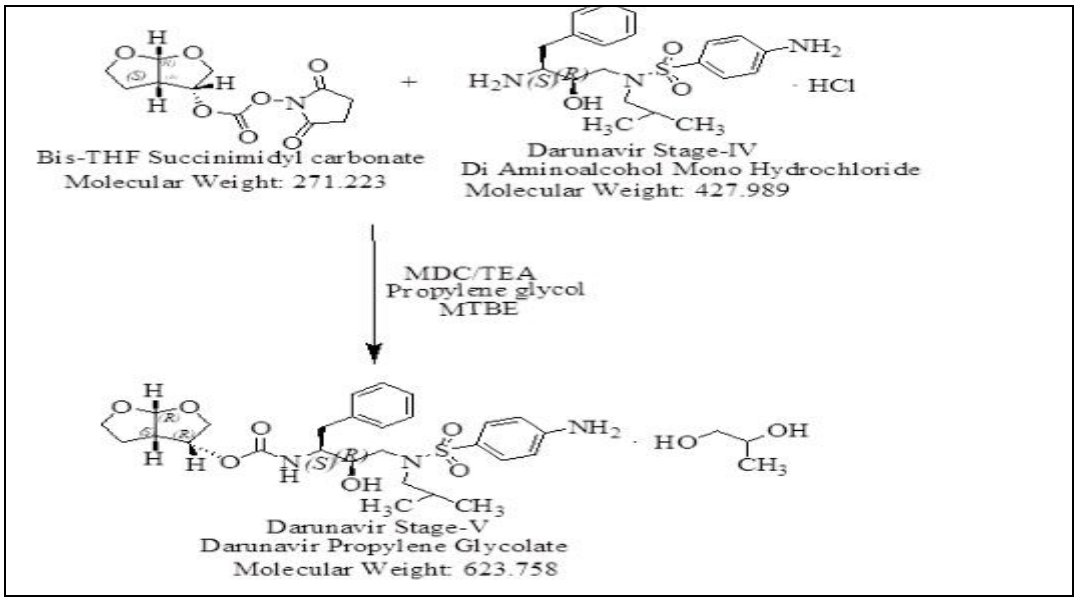
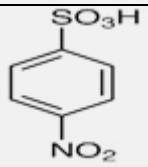
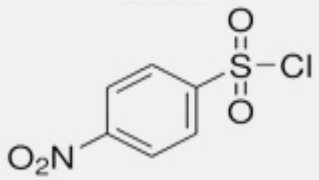
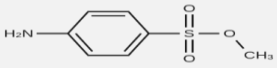


FIG. 2: SCHEMATIC REPRESENTATION OF DARUNAVIR PROPYLENE GLYCOLATE

TABLE 1: GTI (GENOTOXIC IMPURITIES) OF DARUNAVIR PROPYLENE GLYCOLATE DRUG SUBSTANCE

S. no.	Name of Compound	Structure of the impurity
1	4- Nitro benzene sulfonoic acid	
2	4-Nitrobenzenesulfonyl chloride	
3	Methyl-4-amino benzene sulfonate	

Material and Methods / Experimental Details / Methodology:

Materials and Methods: Reagents are used the HPLC grade. Ammonium Bicarbonate (NH_4HCO_3), Acetonitrile taken commercially from Merck. Darunavir propylene glycolate and impurities procured from different sources. Purified water is used in house and polytetrafluoroethylene (PTFE) membranes with a pore size of 0.45 µm has been used to filtration of the mobile phase.

An Ultra-performance liquid chromatography (UPLC), Acquity H-Class system, with a gradient mixer assembly, Sample Manager - F.T.N (Flow through needle) auto injector, with a column oven coupled to Xevo TQS Triple Quadrupole LC/MS/MS Mass Spectrometer, (Make Waters).Column was employed in the method was UPLC BEH C18, 1.7 µm (100×2.1mm) all the weighing in the experiments was done with Sartorius balance capable of measuring with an accuracy of 0.01 mg [5-10].

Methodology:

Preparation of Standard Solution:

Preparation of 4-Nitrobenzene Sulfonic Acid and Methyl 4-Amino Benzene Sulfonate (Standard Stock Solution):

Stock Solution – I: Weighed 7.0 mg of 4-Nitrobenzene sulfonic acid and 7.0 mg of Methyl 4- Amino benzene sulfonate transferred in to each 50 ml volumetric flask respectively and added 30 ml of diluent was added to each flasks and sonicated for 25 mins and made up to the mark with diluent it was used as a primary standard stock solution A and B solution.

Intermediate Stock Solution -I (280.0 µg/g): 1ml from standard stock solution -A was pipetted out and taken into a 50ml volumetric flask and made up with diluent.

Intermediate Stock Solution -II (5.6 µg/g): 1ml from standard stock solution – B was pipetted out and taken into a 50ml volumetric flask and made up with diluent.

Preparation of Standard (1.12 µg/g): From the Intermediate Stock solution -II, 2.0 ml was pipetted out into a 10 ml volumetric flask and made up to mark with diluent.

Preparation of System Suitability Solution: The standard solution was used as a system suitability Solution.

Preparation of Sample Solution: 100 mg of sample was weighed and transferred in to a clean, dry 10 ml volumetric flask and made up to the mark with diluent.

LC-MS Operating Conditions: Mobilephase-A was ammonium bicarbonate and Mobilephase-B was Acetonitrile (ACN) at constant flow rate and injection volumes are 0.3 ml/min and 2.0 µL, respectively.

The gradient programme with run time selected 12 minutes and diluent was used 50:50, %v/v ratio of the acetonitrile and purified water. Gradient programme is shown in the below **Table 2** and MRM shown in shown in **Table 3.**

TABLE 2: GRADIENT PROGRAMME FOR THE MENTIONED METHOD

Time (min)	Mobile Phase A	Mobile Phase B
$T_{0.01}$	70	30
$T_{3.0}$	70	30
$T_{4.0}$	20	80
$T_{8.0}$	20	80
$T_{8.2}$	70	30
$T_{12.0}$	70	30

Optimized the liquid chromatographic conditions and adapted for the identification and quantification of the GTI impurity in the Darunavir propylene glycolate API. Several analytical trails have been done by using different mobilephase buffer with various composition of ACN, and water with different phase of columns like C18, C8 and phenyl phase column.

Ammonium bi carbonate buffer mobile phase -A and acetonitrile as a Mobile phase-B and BEH stationary phase column shows good selectivity, sensitivity and separation of GTI impurity from Darunavir propylene glycolate drug substance. The optimized column is UPLC BEH C18, 1.7 µm (100×2.1mm) used for gradient elution at 30°C. flow rate of 0.3 mL/min. The inj. volume is finalized 2.0 µL and the run time 12 min. and diluent considered as in the ration of 50:50 of acetonitrile and water.

TABLE 3: MRM VALUES OF IMPURITIES

Name	Q1Mass (amu)	Q3Mass (amu)	Cone(v)	Collision energy (v)
4- Nitro benzene sulfonoic acid	202.2	138.1	6	20
Methyl-4-amino benzene sulfonate	186.2	171.1	38	18

Method Validation: Method validation study was conducted as per ICH Q2 (R2) guideline for the optimized LC-MS method and study performed for specificity, precision, limit of detection (LOD), Limit of quantification (LOQ), Linearity and Accuracy parameters.

The linearity study was conducted by preparing and analyzing six different levels of concentrations reported the values of Slope, Y-intercept, Correlation coefficient reported from linearity study. Limit of detection (LOD) and Limit of quantification established based on signal to noise ratio method. The detection Limit (DL)

RESULTS AND DISCUSSION:

Method Development and Optimization: The aim of the LC-MS method in this study to develop a specific, sensitive, precise and Accurate analytical method for quantification of these impurities in Darunavir propylene glycolate drug substance with short runtime method.

Method development initiated by using various acidic mobile phases (such as formic acid, trifluoroacetic acid, diflouoroacetic acid, ammoniumformate) and basic mobile phases such as (Ammonia solution, Ammonium bicarbonate) mix with organic modifiers such as Acetonitrile and methanol isocratic mode elution have been tested.

In acidic mobile phase conditions and basic mobile phase conditions Gaussian curve peak shape was not observed. Optimized the liquid chromatographic conditions and adapted for the identification and quantification of the GTI impurity in the Darunavir propylene glycolate API.

Several analytical trails have been done by using different mobile phase buffers with various composition of ACN, and water with different phase of columns like C18, C8 and phenyl phase column. Ammonium bi carbonate buffer mobile phase -A and acetonitrile as a Mobile phase-B and BEH stationary phase column shows good selectivity, sensitivity and separation of GTI impurity from Darunavir propylene glycolate drug substance.

The optimized column is UPLC BEH C18, 1.7 µm (100×2.1mm) used for gradient elution at 30°C. flow rate of 0.3 mL/min. The inj. volume is finalized 2.0 µL and the run time 12 min. and diluent considered as in the ration of 50:50 of acetonitrile and water

In order to develop a simple, sensitive and selective LC-MS/MS method that can separate and quantify three potential genotoxic impurities in the Darunavir propylene glycolate active pharmaceutical ingredient.

A few columns were used to obtain the most appropriate peak shape and separation. While testing an all analyzed impurities. There was a greater overlap between 4- Nitro benzene sulfonic acid& Methyl 4- aminobenzene sulfonate. When using an ACQUI TY BEH C18 column and poor peak shapes were observed when using a Waters Symmetry C18 column.

A UPLC BEH C18, 1.7 µm (100×2.1mm) was found satisfactory and suitable for peak shape and separation, as well as response of analytes. The mobile phase was operated in gradient mode using ammonium bicrbonate in water and acetonitrile. Acetonitrile was tested as a potential organic phase and chosen for its much better elution efficiency.

The flow rate of the mobile phase was maintained at 0.3 mL/min. With the column temperature set at 30°C. The retention time for 4- Nitro benzene sulfonic acid shown in **Fig. 3** and Methyl 4-aminobenzene sulfonate shown in **Fig. 4** were found to 0.71 and 1.99 respectively and the peak corresponding to Darunavir propylene glycolate was eluted at 11.08 min.

The chromatogram is given in the Electron spray ionization negative mode [11-16].

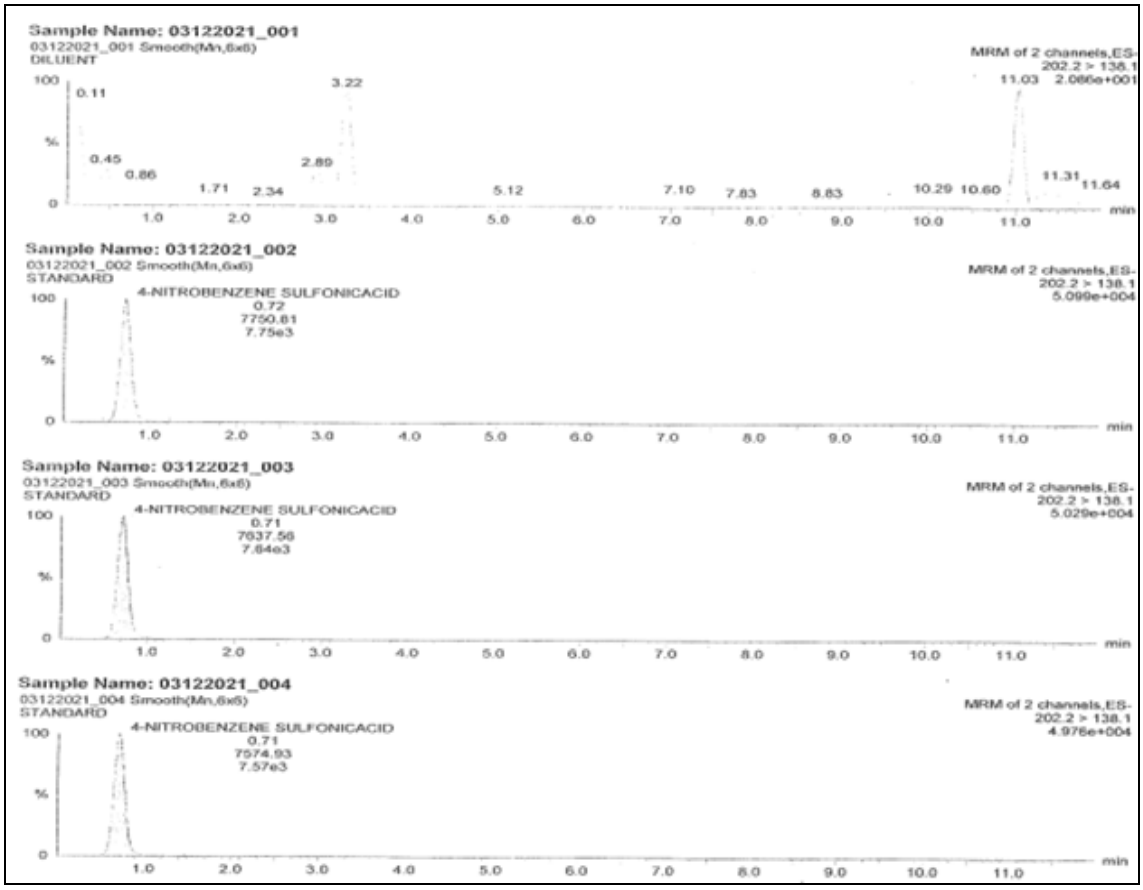


FIG. 3: CHROMATOGRAM OF 4- NITRO BENZENE SULFONIC ACID

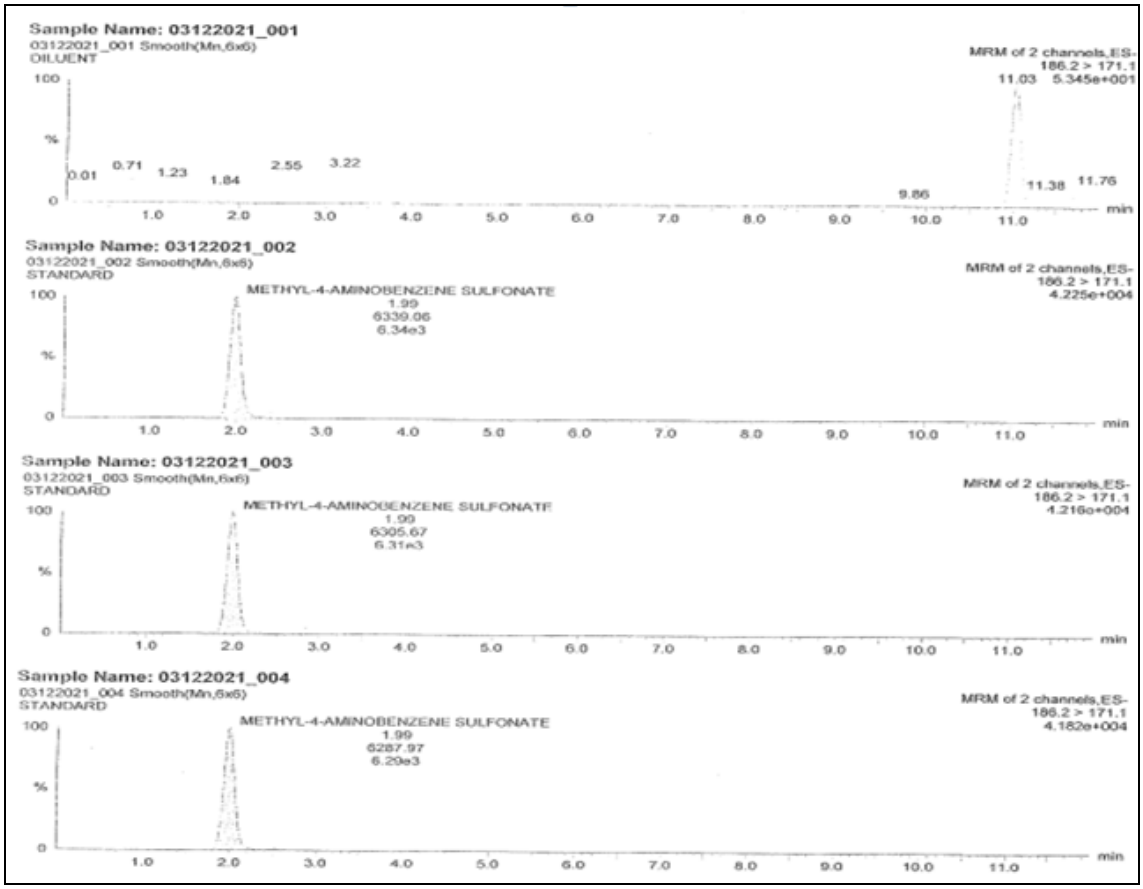


FIG. 4: CHROMATOGRAM OF METHYL 4- AMINOBENZENE SULFONATE

Optimization of MS/MS Conditions: MS Conditions optimization started by using electron spray ionization (ESI) source in negative mode. Source Type: ESI, Mode of Ionization: Negative, Capillary voltage: 2.5 kv, Source Temperature: 150° C, Desolvation temperature: 400° C, Cone gas flow: 150 L/Hr Desolvation gas flow: 1000 L/Hr, Nebulizer (Bar): 6.0, Condition for MRM: Scan Type: MRM, Function type: MRM of 1 channel

Validation Results of the Method: The LC-MS method for trace level quantification of Impurity-A and Impurity-B in Bazedoxifene Acetate drug substance was validated as per ICH Q2(R2) guidelines. The method was evaluated for its specificity, Sensitivity, LOD (limit of detection), LOQ (Limit of quantification), Linearity, Accuracy and Precision

Specificity: Sample solutions of Darunavir propylene glycolate drug substance (control sample), Darunavir propylene glycolate drug substance spiked with and without all the related compounds of 4- Nitro benzene sulfonoic acid and Methyl-4-amino benzene sulfonate were prepared and injected into LCMS/MS, for evaluating specificity of the method and also injected all individual solution for identification purpose. All results meet the acceptance criteria.

Identification: Retention time of spiked shall be comparable with that of standard

Specificity: The interference shall be less than 2.0% of standard response or not more than 20.0% of LOQ response at the respective retention time and MS scan should not show any ion at m/z 202.2 and 186.2 at the retention time of impurities in spiked sample. They should not show similar fragmentation pattern. Specificity results shown in the below **Table 4** and **Table 5.**

TABLE 4: RESULTS FOR IDENTIFICATION

Name	Retention Time		
	Reference Sample	Test Sample	% Difference
4- Nitro benzene sulfonoic acid	0.72	0.70	0.25
Methyl-4-amino benzene sulfonate	1.99	1.98	1.00

TABLE 5: RESULTS FOR SPECIFICITY

Name	Area		MRM Trace
	Control Sample	Spiked Sample	
4- Nitro benzene sulfonoic acid	-	10903	202.2-138.1
Methyl-4-amino benzene sulfonate	-	10749	186.2-171.1

Sensitivity (Precision at LOQ and LOD): The LOD and LOQ were calculated based signal to noise (S/N) ratios method. LOD and LOQ values were tabulated in **Table 6**. Precision at LOD & LOQ shown in **Table 7** (0.11and 0.22 µg/g by considering the sample concentration) was performed by preparing six individual preparations of both impurities, % RSD results were well within the acceptance criteria. Accuracy at LOQ level was performed and recovery results found to be well within the acceptance criteria [17].

TABLE 6: LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION RESULTS

S. no.	Impurity Name	LOD		LOQ	
		Concentration	S/N Ratio	Concentration	S/N Ratio
1	4- Nitro benzene sulfonic acid	0.11 µg/g	360.2	022 µg/g	743.7
2	Methyl 4-aminobenzene sulfonate	0.11 µg/g	152.6	0.1 µg/g	394.3

TABLE 7: PRECISION RESULTS OF LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

	4- Nitro benzene sulfonoic acid		Methyl -4-amino benzene sulfonate	
Injection ID	LOD	LOQ	LOD	LOQ
1.	729	1499	716	1508
2.	719	1424	723	1514
3.	739	1463	702	1533
4.	701	1409	760	1487
5.	713	1479	767	1546
6.	724	1432	703	1559

Statistical analysis					
Mean	721	1451	Mean	729	1525
SD	13.15	34.89	SD	28.33	26.51
%RSD	1.8	2.4	%RSD	3.9	1,7

Linearity: The series of dilutions were prepared, which is directly proportional to the concentration of the analyte in the sample solutions at concentration between from 0.23 µg / g to 1.69 µg / g for the both the impurities to prove the linearity of analytical procedure is its ability to obtained the results. Drawn the graph shown in **Fig. 5** and **Fig. 6**. Between peak responses and analyte concentration in (µg /g). Slope, intercept, and correlation coefficient values were obtained from the graph. The correlation coefficient for two impurities has been found 0.999 and the results were tabulated in the below table 8 and linearity shown in **Fig. 5** and **Fig. 6.**

TABLE 8: LINEARITY OF 4-NITRO BENZENE SULFONIC ACID AND METHYL-4-AMINO BENZENE SULFONATE

4- Nitro benzene sulfonoic acid				Methyl -4-amino benzene sulfonate		
S. no.	**Concentration (µg/g)**	**Area**	**% Accuracy**	**Concentration (µg/g)**	**Area**	**% Accuracy**
1	0.23	1744	98.8	0.23	1537	100.2
2	0.56	4400	101.3	0.56	3866	100.9
3	0.84	6599	101.1	0.84	5693	99.5
4	1.13	8680	98.7	1.13	7509	98.6
5	1.41	11012	100.3	1.41	9514	100.1
6	1.69	13137	99.8	1.69	11495	100.8
Slope: 7807 Intercept: 29.41 Correlation Coefficient :0.9998				Slope: 6756 Intercept: 48.31 Correlation Coefficient: 0.9999		

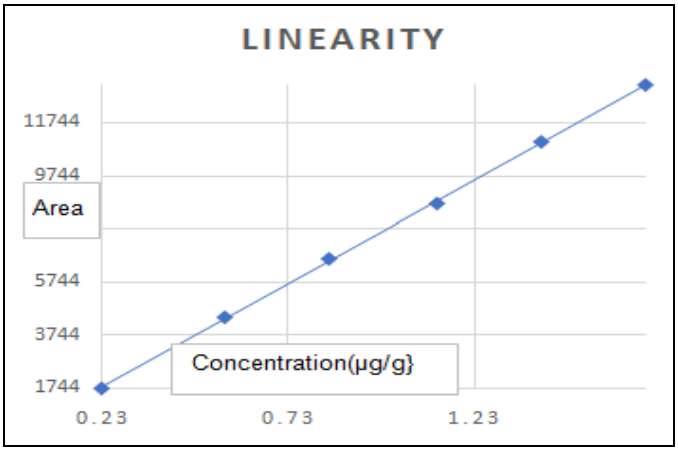


FIG. 5: LINEARITY GRAPH OF 4- NITRO BENZENE SULFONOIC ACID

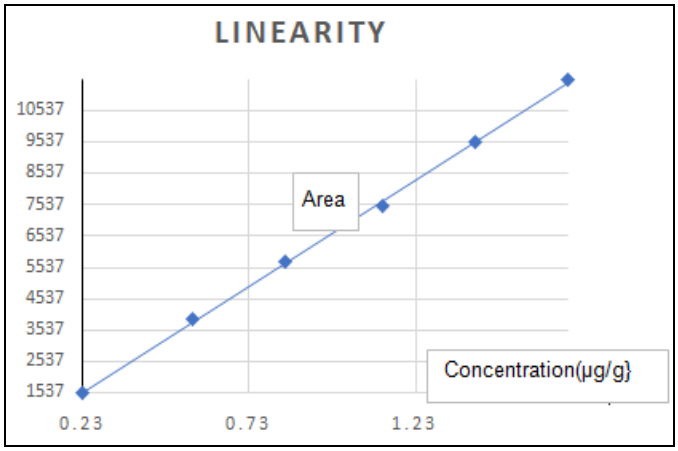


FIG. 6: LINEARITY GRAPH OF METHYL -4-AMINO BENZENE SULFONATE

Precision:

Method Precision: Method precision was performed by spiking the GTI impurities at 100% level with Darunavir propylene glycolate API for six replicated preparations and injected into system. Calculated the % RSD for the content of GTI impurities in the spiked sample. Precision at 100% level was performed by spiking the GTI impurities to the diluent six times. Each solution was injected once and % RSD was calculated for the content of GTI impurities. The calculated % RSD for three levels is between 5.4% and 3.2 % respectively. Results were within an acceptable range and shown in below **Table 9** and **Table 10.**

TABLE 9: RESULTS FOR SYSTEM PRECISION

Impurity Name	**Area**					
	Inj-1	**Inj-2**	**Inj-3**	**Inj-4**	**Inj-5**	**Inj-6**
4-Nitro benzene sulfonic acid	8347	7846	7863	7890	7390	7152
Methyl-4-amino benzene sulfonate	6958	6724	6706	7021	7028	7217

Statistical analysis			
Impurity Name	**Mean**	**STDEV**	**% RSD**
4- Nitro benzene sulfonic acid	7748	420.76	5.4
Methyl -4-amino benzene sulfonate	6972	223.86	3.2

TABLE 10: RESULTS FOR METHOD PRECISION

Impurity Name	**Method precision (µg/g)**					
	Prep-1	**Prep-2**	**Prep-3**	**Prep-4**	**Prep-5**	**Prep-6**
4-Nitro benzene sulfonic acid	1.22	1.14	1.14	1.15	1.08	1.04
Methyl-4-amino benzene sulfonate	1.23	1.19	1.19	1.24	1.28	1.28
Statistical analysis						
Impurity Name	**Mean**		**STDEV**			**% RSD**
4- Nitro benzene sulfonic acid	1.13		0.06			5.5
Methyl -4-amino benzene sulfonate	1.24		0.04			3.3

Intermediate Precision: Multiple samples were taken from a sample stock solution, and six working sample solutions of the same concentrations were prepared. Each injection from each working sample solution was given on the following day of the sample preparation, and the obtained areas are listed in **Table 11.** The average area, standard deviation, and % RSD for the found to be 5.6%, and 2.2% for Nitro benzene sulphonic acid and Methyl-4-amino benzene sulfonate respectively. Because the accuracy limit was lies between 80.0 % and 120.0 %. The values are shown in below **Table 11.**

TABLE 11: METHOD PRECISION AND INTERMEDIATE PRECISION RESULTS

Method precision (µg/g)						
4- Nitro benzene sulfonoic acid	1.22	1.14	1.14	1.15	1.08	1.04
Methyl-4-amino benzene sulfonate	1.23	1.19	1.19	1.24	1.28	1.28
Intermediate precision (µg/g)						
4- Nitro benzene sulfonoic acid	1.16	1.21	1.19	1.18	1.13	1.03
Methyl-4-amino benzene sulfonate	1.06	1.12	1.09	1.10	1.12	1.12
Over all Statistical analysis (Method and intermediate precision)						
Name	**Overall mean**		**Overall SD**		**Overall RSD**	
4- Nitro benzene sulfonoic acid	1.14		0.06		5.3	
Methyl-4-amino benzene sulfonate	1.17		0.08		6.8	

Accuracy: From the recovery studies the accuracy of method was determined. In this process the recovery studies were performed in triplicate at LOQ level, 100% and 150%. The percentage recoveries were calculated to verify the method accuracy. The recovery values for - Nitro benzene sulfonic acid (98.55-103.7%), Methyl -4-amino benzene sulfonate (98.8 - 103%). and the values are reported in below **Table 12.**

TABLE 12: RECOVERY RESULTS

Level	**4- Nitro benzene sulfonoic acid**			**Methyl -4-amino benzene sulfonate**		
	Area of spiked sample	**Area of standard**	**% recovery**	**Area of spiked sample**	**Area of standard**	**% recovery**
LOQ Level	7809	7787	98.0	11798	11714	99.0
100%	7832	8072	98.3	12452	12049	104.5
150%	7913	8046	98.3	12818	11985	107.6
Statistical analysis						
Mean area of standard			7968.3		11916	
Mean% Recovery			98.5		103.7	

Robustness: Robustness conditions such as flow minus (0.27 ml/min), flow plus (0.33ml/min), mobile phase minus (55:45v/v), mobile phase plus (45:55 v/v), temperature minus (25°C), and temperature plus (35°C) were maintained, and samples were injected induplicate. The % RSD was calculated and determined to be within the acceptable range. Results are tabulated below **Table 13.**

TABLE 13: ROBUSTNESS RESULTS ARE SHOWN IN BELOW TABLE

Results		
Low flow (0.27ml/min)		
Injection ID	**4- Nitro benzene sulfonoic acid**	**Methyl -4-amino benzene sulfonate**
1.	10740	10755
2.	11065	10753
3.	11296	10689
4.	11121	11011
5.	11414	10831
6.	11487	11145
Statistical analysis		
Mean	11187	10864
SD	272.93	176.91
%RSD	2.4	1.6
High flow(0.33ml/min)		
Injection ID	**4- Nitro benzene sulfonoic acid**	**Methyl -4-amino benzene sulfonate**
1.	8582	7720
2.	7912	7770
3.	8458	7926
4.	8059	7696
5.	8138	7437
6.	8310	7759
Statistical analysis		
Mean	8243	7718
SD	253.01	159.44
%RSD	3.1	2.1
Source cleaning before		
Injection ID	**4- Nitro benzene sulfonoic acid**	**Methyl -4-amino benzene sulfonate**
1.	7751	6339
2.	7638	6306
3.	7575	6288
4.	7874	6389
5.	7728	6310
6.	7597	6351
Statistical analysis		
Mean	7694	6331
SD	112.70	36.72
%RSD	1.5	0.6
Source cleaning after		
Injection ID	**4- Nitro benzene sulfonoic acid**	**Methyl -4-amino benzene sulfonate**
1.	10879	7960
2.	11208	7887
3.	10756	8315
4.	10897	7860
5.	10891	7958
6.	11077	8058
Statistical analysis		
Mean	10951	8006
SD	162.26	166.18
%RSD	1.5	2.1

Summary

Parameter	**Variation**	**4-Nitrobenzene sulfonic acid**		**Methyl-4-aminibenzene sulfonate**	
		RT(min)	**%RSD**	**RT(min)**	**%RSD**
Flow rate	+10	0.78	2.4	2.20	1.6
	-10	0.64	3.1	1.80	2.1
Source cleaning	Before	0.72	1.5	1.99	0.6
	After	0.71	1.5	1.98	2.1

The system suitability results at each of the varied conditions complied the requirement as per the test procedure. Hence it can be concluded that the test method is robust across the extent of changes studied for each of the above parameter [18].

CONCLUSION: A novel selective, highly sensitive and hyphenated analytical method using LC-MS/MS coupled with negative electrospray ionization has been developed for quantification of genotoxic impurities like Nitro benzene sulfonic acid and Methyl-4-amino benzene sulfonate at in Darunavir propylene glycolate API. MRM mode, in comparison, offered improved selectivity and sensitivity for the analyte's screening and quantification. When used in MRM mode, the matrix effects that limit precision and LOD and LOQ levels are drastically reduced or eliminated. The method has advantages over previously described methods due to its increased sensitivity and easier sample preparation process. For its potential use with additional drug compounds, this technique might be further researched. This method is completely validated and shows good linearity, accuracy, repeatability and robustness. The above developed method was very effective for the determination of Nitro benzene sulfonic acid and Methyl-4-amino benzene sulfonate in Darunavir propylene glycolate API.

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