Toxicity estimation using QSAR methodology and analytical approach for the accurate determination of two potential genotoxic impurities in Abacavir Sulfate antiviral drug at TTC level by liquid chromatography-mass spectrometry (LC-MS/MS)

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Abstract

Toxicity estimation of N-(2-amino-4,6-dichloropyrimidin-5-yl) formamide (FADCP) and 2-Amino-5-Nitroso Pyrimidine-4,6-diol (N NITROSO) impurities were done using Case ultra-software and both the impurities found Mutagenic under the class 3. The sensitive analytical method was established and validated for the potential genotoxic impurities, FADCP, and N NITROSO in the Abacavir Sulfate drug sample using Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Zorbax SB Phenyl (250mm x 4.6mm, 5.0µm) column was used to quantify the genotoxic impurities using LC-MS/MS. Formic acid (0.1%) was used as mobile phase A and acetonitrile (LCMS grade) was used as mobile phase B in gradient mode. The flow rate and run time of the developed quantification method were 0.5 mL/min and 45minutes. A tandem mass spectrometer consisting of triple quadrupole coupled with an electrospray ionization source, multiple reaction monitoring targeted approach of LC-MS/MS with the positive ionization mode was used to quantify the potential genotoxic impurities. The linearity range covered in the developed method was 0.76ppm to 3.79ppm for N-(2-amino-4,6-dichloropyrimidin-5-yl) formamide (FADCP) and 0.75ppm to 3.76ppm for 2-Amino-5-nitroso Pyrimidine-4,6-diol (N NITROSO) with respect to Abacavir Sulfate drug sample concentration with a correlation coefficient 1.000 & 0.9996 respectively. The accuracy of the method was in the range of 101.73% to 104.33% for FADCP impurity and 111.68% to 98.66% for N NITROSO impurity. This developed LC-MS/MS method is validated as per the International Council on Harmonization (ICH) guidelines and is able to quantify the potential genotoxic impurities at minimal levels.

Keywords: Abacavir Sulfate, QSAR, Method Validation, FADCP, N NITROSO.

INTRODUCTION

Abacavir sulfate API chemical name is ((1S, 4R) Amino-6-(Cyclopropylamine)-9H-purin-9-yl) cyclopentenyl) methanol Hemisulfate. Abacavir sulfate is a nucleoside reverse transcriptase inhibitor (NRTIs). Abacavir sulfate is used as either a 600 mg once-daily or 300 mg twice-daily in combination with other drugs. These drugs are used in the treatment of human immunodeficiency virus (HIV) infection[9,10].

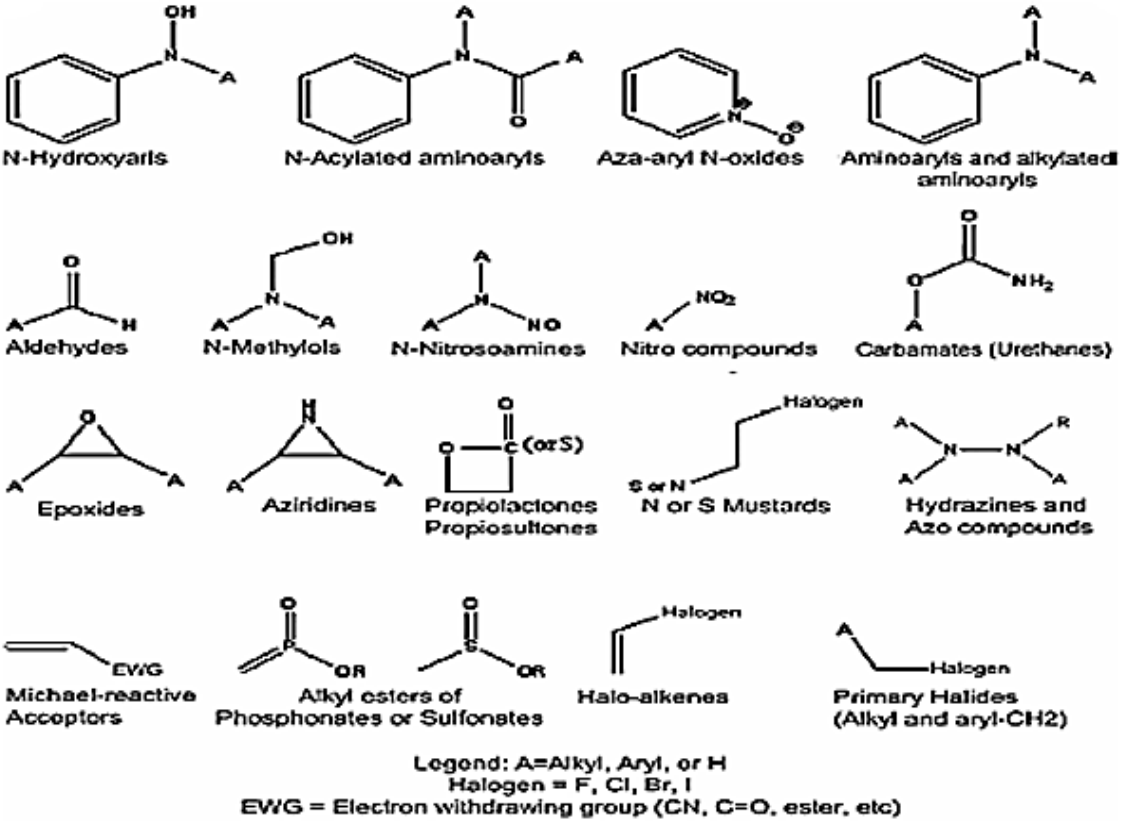
Literatures available for quantification of potential genotoxic impurities namely, N',N'''-(4,6-dichloropyrimidine-2,5-diyl)bis[N,N-dimethyl(imidoformamide) (Impurity-I), {N-(2-amino4-chloro-6-{[(1S,2R)-2-(hydroxymethyl)cyclopent-3-en-1- yl]amino}pyrimidin-5-yl)formamide} (Impurity-II) and {(1R,5S)-5- [(2,5-diamino-6-chloropyrimidin-4-yl)amino]cyclopent-2-en-1-yl} methanol (Impurity-III) and 2,5-Diamino-4,6-dichloro pyrimidine in Abacavir Sulfate [1,2]. Literatures reported related to Abacavir Sulfate forced degradation studies and chromatographic method development of Abacavir Sulfate and its impurities[3,4,5,6,7]. Quantitative determination of FADCP impurity as a related substances in Abacavir Sulfate by HPLC was carried out but determination of impurity was 0.1% with respect to Abacavir Sulfate drug concentration was done[9]. No method has been available for the determination of FADCP and N NITROSO genotoxic impurities in Abacavir Sulfate active pharmaceutical ingredient by LCMS-MS with limit not more than 2.5 ppm with respect to sample.

By doing changes in natural processes or by chemical reactions, the active pharmaceutical ingredients are manufactured in various pharmaceutical organizations. Manufacturing process reactions includes different types of reagents, intermediate and catalyst. Output of the chemical reaction gives various impurities and these impurities are may be by products of chemical reaction, reagents and intermediate. Formation of impurities are very common in small amount in chemical reaction during synthesis process of active pharmaceutical ingredients.

Impurities formation during synthesis process may be toxic and these impurities may display genotoxicity or carcinogenicity. Therefore, the existence of an impurities in active pharmaceutical ingredients has a worry for regulatory agencies and pharmaceutical manufactures.

This type of potential genotoxic impurities tends to respond with Deoxyribonucleic acid (DNA) and creating carcinogenic threat in patients. Identification of impurities genotoxicity can be done with the help of toxicological software available or alerting functional group are available in structure as per Figure one.

FIGURE 1: STRUCTURAL ALERTS FOR PHARMACEUTICAL IMPURITIES



Structural alerts for pharmaceutical impurities

Potential genotoxic impurities are the impurities which gives structural alerts after toxicological software evaluation and accountable for the cancer in human being.1.5 µg/day is the everyday dose recommendation for the potential genotoxic impurities as per the regulatory agencies' guidelines.

IMPURITIES STRUCTURE TOXICOLOGICAL EVALUATION/PREDICTION BY ULTRA CASE SOFTWARE:

Quantitative Structural Activity Relationship (QSAR) analysis was considered to evaluate the toxicological potential along with the safety-based limit of the impurity following International Conference on Harmonization guideline M7 (R2).

As per the International Conference on Harmonization guideline M7 on Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, need to perform computational toxicology assessment to predict the outcome of a bacterial mutagenicity assay using two different methodologies.

Two different methodologies are expert rule-based and statistical-based. Both mentioned Methodologies are prediction methodologies and (Q)SAR models are working according to these methodologies. Organization for Economic Co-operation and Development (OECD) also set some validation principles for the computational toxicological prediction and (Q)SAR utilizing those principles for toxicological evaluation.

N NITROSO impurity and FADCP impurity toxicological estimation done by Case ultra-software or model. Case ultra-software or model is expert rule-based and statistical based predictive toxicology software program that makes qualitative estimations of endpoint risk. A computer program having a knowledge-based system that contains expert knowledge rules in toxicology and applies the rules to make predictions about the toxicity of chemicals. The risk estimation is qualitative, and it were categorized, as 'certain', 'probable', 'plausible', 'equivocal', 'doubted', 'improbable' or 'impossible'. A negative prediction for bacterial in vitro mutagenicity, where an 'inactive' call can be made and non-alerting compounds are evaluated to identify unclassified and misclassified features. Rule based technique involves analysis of several molecular fragments of interest in relation to the variation in biological response on the basis of pre-defined chemical rules and also considering cross-terms fragment descriptors, which could be helpful in identification of key fragment interactions in determining variation of activity.

Case ultra-software or model uses QSAR models in statistical based evaluation to provide a quantitative predictive probability for the potential toxicity of chemicals. The algorithm based *in silico* system is based on well-defined mutagenicity structural alerts from the literature. The computed probability for mutagenesis was corresponding to the input structure sub model.

After evaluation by Case ultra -model, FADCP and N NITROSO (Figure Two) impurities suggested outcome (Table-One) was positive.

FIGURE 2: FADCP, N NITROSO AND ABACAVIR SULFATE CHEMICAL STRUCTURES

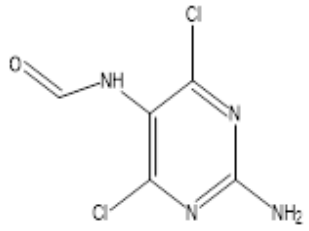
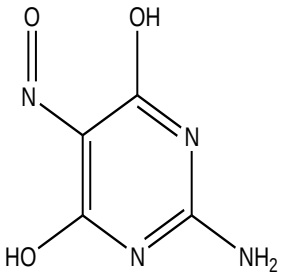
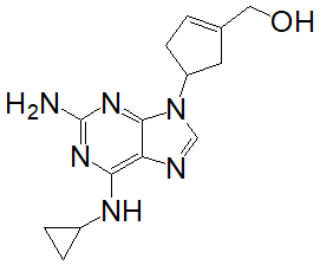
FADCP IMPURITY	N NITROSO IMPURITY	ABACAVIR SULFATE
		

TABLE 1: FADCP AND N NITROSO IMPURITIES TOXICOLOGICAL OUTCOME SUMMARY

Impurity Name	**Genotoxic Predictions as per OECD 471 Bacterial Mutagenicity Model**	**Genotoxic Predictions as per Expert Rules for Bacterial Mutagenicity Model**	**Classification**
FADCP IMPURITY	Outcome: Positive Calculated Probability: 62.1%	Outcome: Positive Calculated Probability: 75.9 %	Class 3 (Mutagenic)
N	Outcome: Positive	Outcome: Positive	Class 3

NITROSO IMPURITY	Calculated Probability: 86.6%	Calculated Probability: 94.9 %	(Mutagenic)

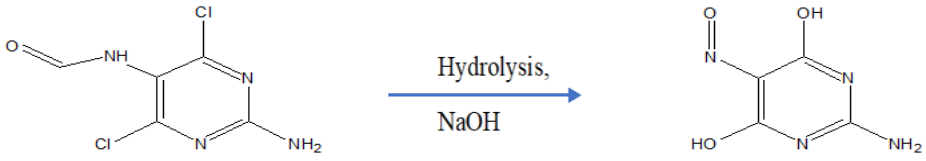
As per the TTC (Threshold of Toxicological Concern) calculation based on the daily dose of Abacavir Sulfate drug (600 mg/day dose), both potential genotoxic impurities present in the Abacavir sulfate drug should not be more than 2.50 ppm. So, both the genotoxic impurities need to be control to an acceptable level of 2.50 ppm with respective to Abacavir Sulfate drug concentration. To quantify the potential genotoxic impurities at small amount or level, highly sensitive analytical instrument is required. Genotoxic impurities determination analytical method development at small amount using analytical instruments like ultraviolet-visible spectrophotometry, gas chromatography, high-performance liquid chromatography are not useful in the pharmaceutical industry due to the low sensitivity. Low sensitivity can be achieved by highly sensitive analytical instrument like LC-MS/MS, GCMS etc. Most of the authors used MS technique for the quantification of potential genotoxic impurities because of the reliability and sensitivity of LC-MS/MS method.[14,15,16,17]

So, considering 2.50 ppm is the quantification limit of both the potential genotoxic impurities with respect to Abacavir sulfate drug sample concentration, this analytical method was developed using LC-MS/MS analytical approach.

SOURCE OF IMPURITIES:

During the manufacturing process of Abacavir Sulfate, FADCP is used as an intermediate in first stage along with sodium hydroxide solution. There is a probability of formation of N NITROSO impurity when FADCP undergoes hydrolysis reaction in presence of basic condition (Figure Three).

FIGURE 3: POSSIBLE REACTION MECHANISM FOR THE FORMATION OF N NITROSO IMPURITY



MATERIALS AND METHODS:

Chemicals & Regents:

Mass Spectrometer grade, Acetonitrile and Formic acid were obtained from Biosolve (Mumbai, India). Purified water was collected from Milli Q Water System (Millipore, USA). Abacavir Sulfate API sample received from manufacture was a gift sample and genotoxic impurities were obtained from impurities trader in Mumbai, India. Ammonia solutions 25% (Merck Germany) were obtained from Merck.

Analytical Instrumentation:

Applied Biosystems AB Sciex 5500 model (Made in Singapore) LC-MS/MS system along with the high-performance liquid chromatography consisting of an Agilent 1290 Infinity Binary pump(G4220A), an Agilent 1290 Infinity DAD detector(G4212A), an Agilent 1290 Infinity sampler(G4226A), an Agilent 1290 Infinity column Thermostat(G1316C) (Made in Germany) were used for analytical method development for potential genotoxic impurities and further validation purpose. For data acquisition and processing, Analyst 1.6.2 software was used on a Dell computer. Analytical column was Zorbax SB

phenyl (250mmX4.6mm, 5.0 µm) (Agilent Technologies, Germany) was used for analytical method development and validation.

Gradient mode applied using mobile phase A (0.1% Formic acid in Water) and mobile phase B (Acetonitrile 100% *v/v*). 0.5 mL/min flow rate of mobile phase was applied, and flow splitter was used to split the flow so that mobile phase flow split down to 0.3 mL/min into the mass spectrometer. 55°C was the column oven temperature and 5°C was the cooler temperature maintained. 10µL was the injection volume. To quantify the potential genotoxic impurities in Abacavir Sulfate, electrospray ionization source was used. Multiple reaction monitoring (MRM) with positive approach of ionization technique was employed in mass spectrometer method. FADCP impurity was observed with its molecular ion $[M+H]^+$ m/z 207.0 and daughter/fragment ion m/z 171.0 (207.0-171.0) in developed method. N NITROSO impurity was observed with its molecular ion $[M+H]^+$m/z 157.0 and fragment/daughter ion m/z 96.0 (157.0 -96.0). Declustering potential was reserved 120 V and entrance potential was reserved 6 V for FADCP impurity and Declustering potential was reserved 50 V and entrance potential was reserved 10 V for N NITROSO impurity whereas the ion spray voltage (V) maintained as 5500V. Nebulization pressures (psi) namely ion source gas 1 and ion source gas 2 were continued at 30 and 60 respectively. Developed analytical method is useful for the quantification of above mentioned both the genotoxic impurities in Abacavir Sulfate active pharmaceutical ingredient using LC-MS/MS technique.

Solution preparation for Standard and Sample

FADCP impurity stock solution was prepared in diluent (Acetonitrile: Water (50:50 *v/v*)) at 0.05 mg/mL.0.05 mg/mL stock solution of N NITROSO impurity was first prepared in 10 ml of 0.1% Ammonia Solution (25% V/V) and then further diluted with diluent (Acetonitrile: Water (50:50 *v/v*). Then diluted stock solution of 0.0005 mg/mL prepared for FADCP and N NITROSO impurities by diluting 0.50 mL of the 0.05 mg/mL solutions of FADCP and N NITROSO impurities to 50 mL with diluent.

Prepared final standard solution by diluting the 0.50mL of diluted stock solution of FADCP and N NITROSO impurity into 10 mL with diluent at 2.50ppm with respect to Abacavir sulfate concentration. 10 mg/mL, Abacavir Sulfate sample solution was prepared by dissolving appropriate amount of Abacavir Sulfate in the diluent. Solutions for recovery studies were prepared at 0.75ppm, 2.50ppm and 3.75ppm by diluting the above diluted stock solution with the required amount of diluent for both the potential genotoxic impurities. Linearity solutions were prepared for linearity study at 0.75, 1.25, 2.50, 3.00 and 3.75ppm by diluting the above diluted stock solution with necessary amount of diluent. All the prepared standards solutions were sonicated before the analysis.

Analytical method development:

The current method was established by trying different column chemistry stationary phases to complete significant separation of the potential genotoxic impurities along with Abacavir Sulfate drug substance. Genotoxic impurities structures are analogues with Abacavir Sulfate. To achieve sensitivity and separation between impurities and Abacavir Sulfate along with the recovery within the acceptance range, numerous analytical columns like Waters C18 (250mm × 4.6mm, 5.0µm), Ascentis Express C18 (100mm × 4.6mm, 2.7µm), YMC Triart C18 EXRS (150mm × 4.6mm,5.0µm), Zorbax SB Phenyl (250mm × 4.6 mm, 5.0µm) were evaluated during method development along with different type of mobile phase A. When Zorbax SB Phenyl column was used with 0.1% formic Acid as a mobile phase A and Acetonitrile as a mobile phase in port B with gradient system, the recovery of both the genotoxic impurities was found within the acceptance range.

When 0.02% Ammonia (25% Solution) used as a mobile phase, the all peaks are eluted early on void volume. (Trial 2). YMC Triart column also used on same mobile phase but response of genotoxic impurities was less.

On Zorbax SB phenyl column (250 mm × 4.6mm, 5.0µm), both genotoxic impurities and Abacavir Sulfate were separated with each other. Intensity for both the potential genotoxic impurities were observed satisfactory. Different types of mobile phase polarity utilized with different compositions for analytical method development. Initially tried to set method isocratic but later applied gradient method. Lastly, respectable separation and intensity were observed at a ratio of mobile phase A (0.1 % formic acid) mobile phase B (Acetonitrile (100 *v/v*)) with gradient program. Established gradient program was mentioned in **Table Two** which was more effective in reaching ideal separation of both the genotoxic impurities from each other with respect to

Abacavir sulfate peak. To get the separation between both the genotoxic impurities and Abacavir Sulfate, the column over temperature was maintained at 55°C. Diverted valve was applied during analysis of a sample or spiked sample, to divert the Abacavir Sulfate drug peak into waste from 20 min to 45 min retention time to evade any further interference in mass spectrometer.

TABLE 2: MOBILE PHASE GRADIENT PROGRAM

Time(min)	% Mobile phase A	% Mobile phase B
0.01	95.0	5.0
20	95.0	5.0
22	10.0	90.0
30	10.0	90.0
32	95.0	5.0
45	95.0	5.0

RESULTS AND DISCUSSION:

Method Validation:

The method specificity was proved by injecting the blank solution, individual genotoxic impurities solution, Abacavir Sulfate sample solution and the related chromatograms are accessible in Figure 4-7. The blank solution chromatogram in Figure 4 disclosed that interference was not observed at the retention times of Abacavir Sulfate drug as well as potential genotoxic impurities. Related chromatograms of both the impurities displayed in Figure 4 to 7 showed that FADCP impurity and N NITROSO impurity eluted at the retention times of 16.48 min and 7.0 min respectively. The chromatograms obtained using developed method shows developed method using LC-MS/MS was able to separate effectively both the genotoxic impurities with each other and with Abacavir Sulfate main drug.

FIGURE 4: CHROMATOGRAM BLANK

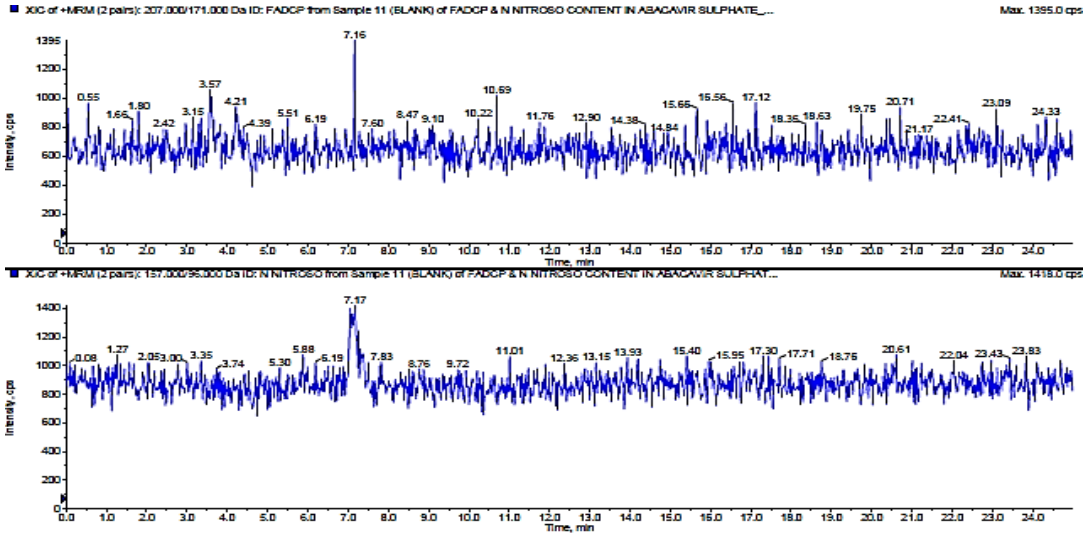


FIGURE 5: FADCP -XTRACTED ION CHROMATOGRAM(XIC)

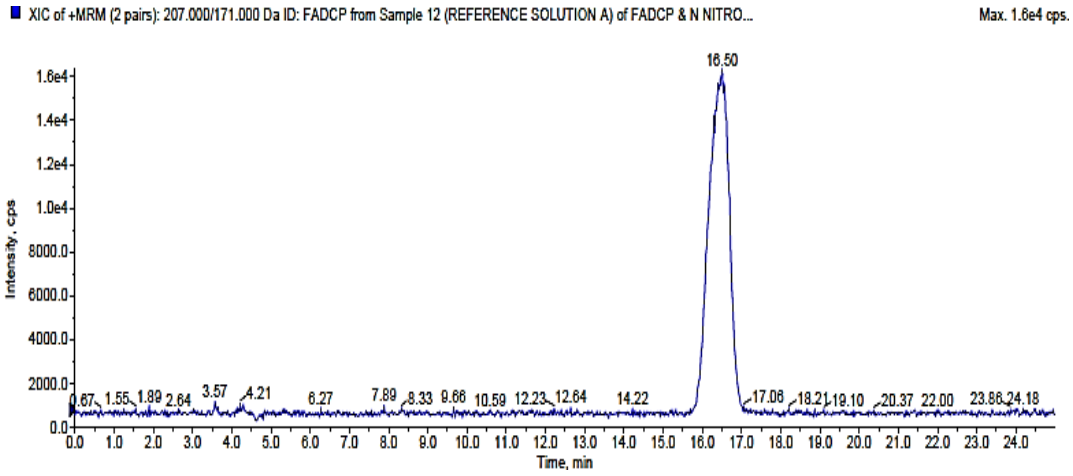


FIGURE 6: N NITROSO -XTRACTED ION CHROMATOGRAM(XIC)

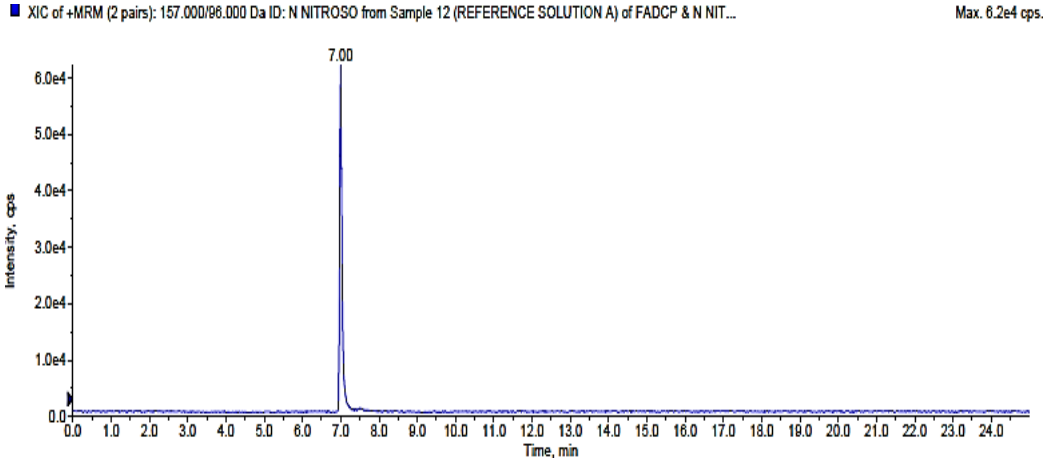
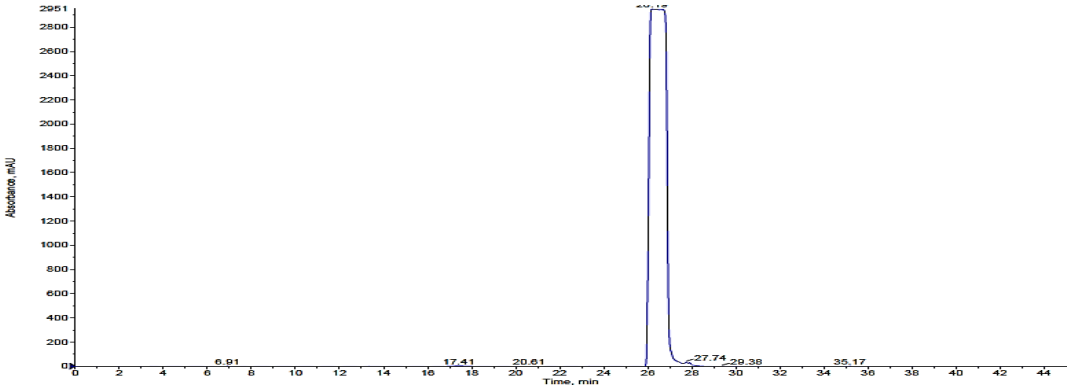


FIGURE 7: ABACAVIR SULFATE UV CHROMATOGRAM



Determined the detection limit (LOD) and quantification limit (LLOQ) for FADCP and N NITROSO impurities from S/N (signal to noise) ratio. Prepared the lower concentrations of potential genotoxic impurities standard solutions to established quantification limit. Solution for quantification limit produced S/N ratio 40 and 69.7 for FADCP and N NITROSO impurities respectively. The LOQ of 0.75ppm is representative for both the impurities with a LOD of 0.25ppm.

FIGURE 8: FADCP S/N RATIO

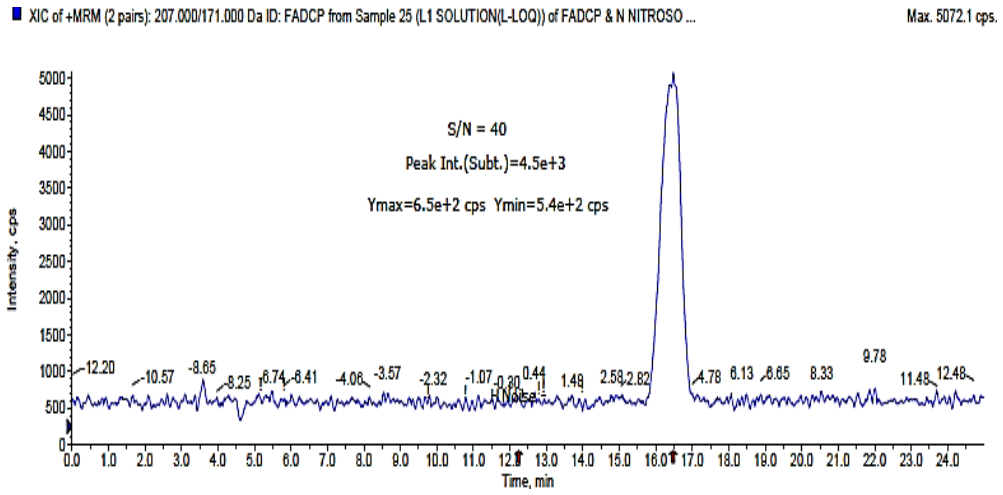
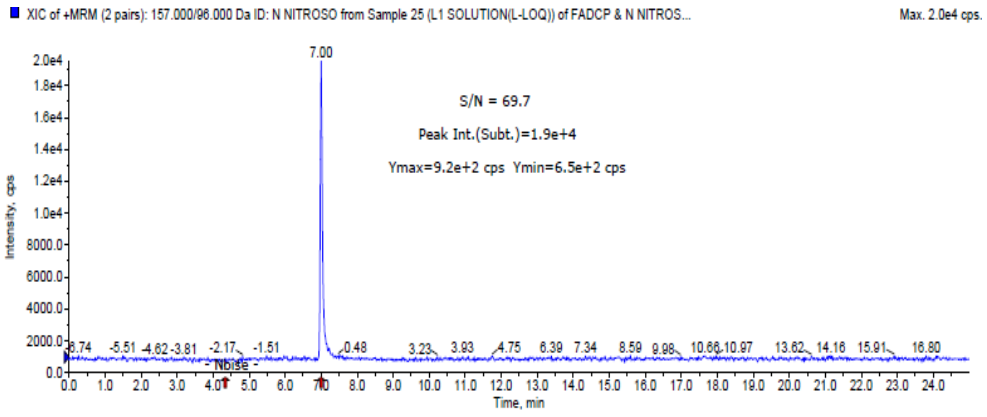


FIGURE 9: N NITROSO S/N RATIO



The developed method linearity in multiple reaction monitoring mode was established by injecting both the potential genotoxic impurities solutions at dissimilar stages of the concentrations between LLOQ (0.75 ppm) and 150% of the target concentration

(3.75ppm). Plotted the calibration curve by drawing the graph between the peak areas observed and concentration of potential genotoxic impurities at 0.75, 1.25, 2.50, 3.00, and 3.75ppm. The slope, intercept and correlation coefficient values found by linear least square regression analysis were accessible in Table Three.

TABLE 3: FADCP AND N NITROSO LINERITY RESULTS

Linearity Levels (L1 to L5)	FADCP Concentration in ppm	FADCP Area	N NITROSO Concentration in ppm	N NITROSO Area
30%(L1)	0.7582	174635.503	0.7510	86679.881
50%(L2)	1.2637	286696.892	1.2517	140598.427
100%(L3)	2.5273	563916.152	2.5034	275546.245
120%(L4)	3.0328	679309.099	3.0041	325179.129
150%(L5)	3.7910	845589.895	3.7551	399572.088
Correlation Coefficient(r^2)		1.000	Correlation Coefficient(r^2)	0.9996
Slope		221289	Slope	104594
Intercept		6692.4	Intercept	9858.1

The method accuracy was determined by spiking study of both the potential genotoxic impurities at LLOQ, 100% and 150% levels of the target concentrations, i.e., 0.75, 2.50, 3.75ppm with respect to Abacavir Sulfate sample concentration.

FIGURE 10: FADCP -XTRACTED ION CHROMATOGRAM(XIC) LLOQ SPIKED SAMPLE

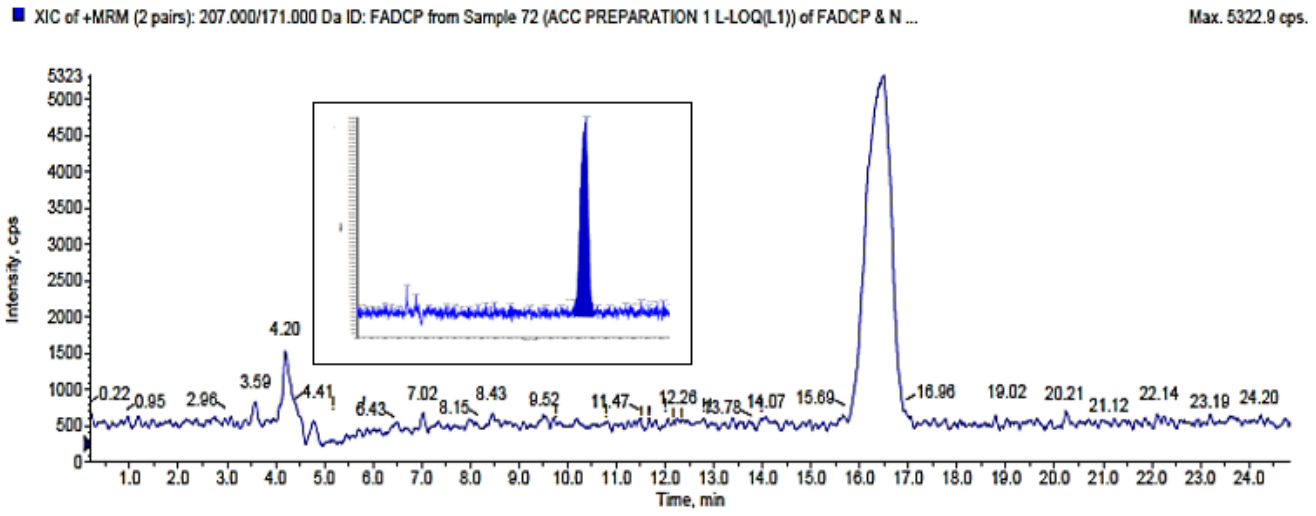
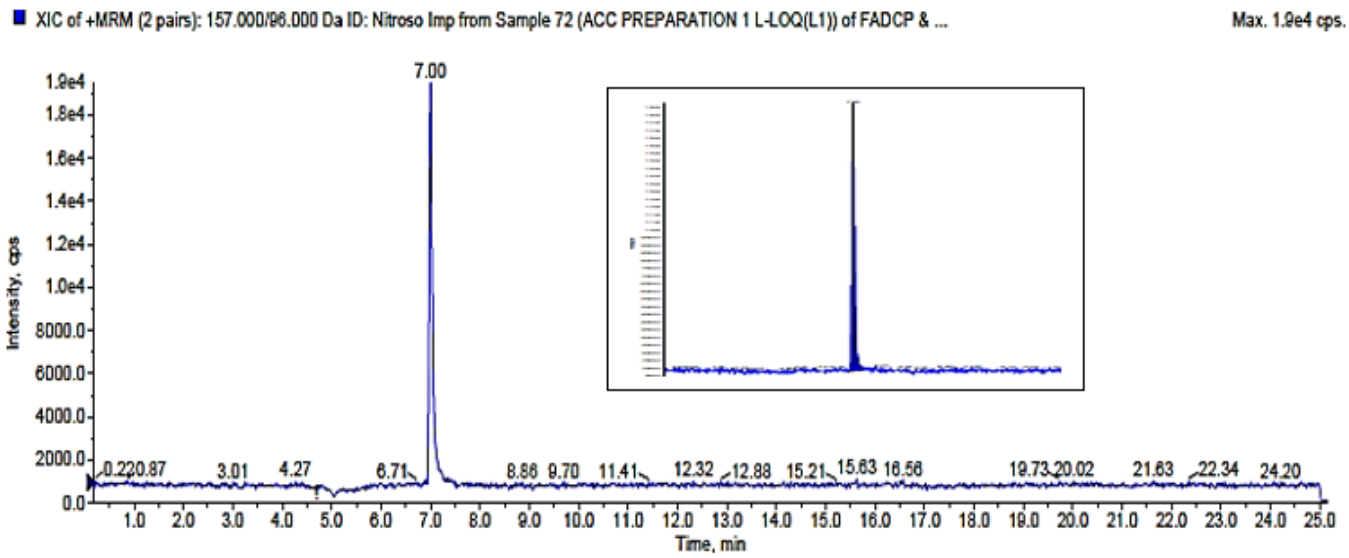


FIGURE 11: N NITROSO -XTRACTED ION CHROMATOGRAM(XIC) LLOQ SPIKED SAMPLE



Accuracy determination was carried out at LLOQ level, Limit level and 150% Level and related data is accessible in Table-Four. The recovery of both the potential genotoxic impurities at three levels (LLOQ level, 100 % and 150 %) should be in between 85.0% to 115.0% and percent relative standard deviation should not be more than 10.0%. Recovery values were found 101.73% to 104.33% for FADCP impurity and 111.68% to 98.62% for N NITROSO impurity with RSD 1.61% & 6.76% respectively.

TABLE 4: RESULTS OF ACCURACY FOR BOTH THE GENOTOXIC IMPURITIES

Impurity Name	**FADCP**			**N NITROSO**		
Level	Theoretical Conc in ppm wrt Sm	Measured Conc in ppm wrt Sm	Recovery %	Theoretical Conc in ppm wrt Sm	Measured Conc in ppm wrt Sm	Recovery %
LLOQ (30%)	0.76	0.77	101.73	0.75	0.84	111.68
100%	2.53	2.56	101.26	2.5	2.52	100.64
150%	3.79	3.95	104.33	3.76	3.7	98.66
	%R.S.D.		1.61	%R.S.D.		6.76

The method precision was determined through repeatability and ruggedness study. Method repeatability was checked by spiking specification level standard concentration in six freshly prepared sample solutions on the same day and RSD of content of each impurity were evaluated and relative standard deviation should not be more than 10.0% are accessible in Table-Five. Relative Standard deviation of FADCP and N NITROSO impurities obtained 0.93% and 1.77% respectively. Precision at LLOQ level was also checked and % relative standard deviation of six replicate injections was 2.31 for FADCP and 2.16 for N NITROSO impurities respectively. Details were available in Table-Six.

Ruggedness was checked by specification level standard concentration of FADCP and N NITROSO impurities in six freshly prepared sample solutions at different day and commutative relative standard deviation of content of each impurity between spike precision and intermediate precision should not be more than 10.0 %. Details were available in Table-Seven.

TABLE 5: RESULTS OF SPIKE PRECISION FOR BOTH THE GENOTOXIC IMPURITIES

Injection	FADCP Imp Concentration obtained in sample	N NITROSO Imp Concentration obtained in sample
1	2.52	2.5
2	2.49	2.41
3	2.49	2.39
4	2.5	2.44
5	2.47	2.48
6	2.54	2.4
Mean	2.5	2.44
S.D.	0.02	0.04
R.S.D.%	0.93	1.77

TABLE 6: RESULTS OF PRECISION AT LLOQ LEVEL

Injection	Area of FADCP Imp Std in LLOQ Solution	Area N NITROSO Imp Std in LLOQ Solution
1	175363.702	84624.999
2	181566.124	87175.66
3	169252.379	84952.285
4	175032.765	85741.907
5	173336.412	88437.69
6	173261.635	89146.745
Mean	174635.503	86679.881
S.D.	4031.743	1871.258
R.S.D.%	2.31	2.16

TABLE 7: RESULTS OF RUGGEDNESS FOR BOTH THE GENOTOXIC IMPURITIES

Injection	FADCP Imp concentration obtained in sample	N NITROSO Imp Concentration obtained in sample
1(Precision)	2.52	2.5
2(Precision)	2.49	2.41
3(Precision)	2.49	2.39
4(Precision)	2.5	2.44
5(Precision)	2.47	2.48

6(Precision)	2.54	2.4
1(Ruguddness)	2.69	2.67
2(Ruguddness)	2.57	2.63
3(Ruguddness)	2.68	2.73
4(Ruguddness)	2.75	2.7
5(Ruguddness)	2.77	2.7
6(Ruguddness)	2.68	2.51
Mean	**2.6**	**2.55**
S.D.	**0.1075**	**0.1307**
% R.S.D.	**4.14**	**5.13**

The method robustness was evaluated by doing deliberately changes in flow rate, column temperature. Effect of the mobile phase flow rate and column temperature changed studied in this parameter. Mobile phase flow rate (0.50 mL/Min) of the developed method was changed by 10 %, i.e., from 0.45 mL/min to 0.55 mL/min and column temperature of the developed method changed from 55ºC to 53ºC and 57ºC. Changes done in developed method in robustness study did not show any substantial changes in separation of potential genotoxic impurities from the Abacavir Sulfate and on chromatographic demonstration.

To demonstrate the stability of FADCP, N NITROSO impurities analytical solutions, target level impurities solution with respect to Abacavir Sulfate drug concentration spiked in the Abacavir Sulfate sample solution and kept at room temperature (25ºC) for 24 hrs. Solution stability was estimated by calculating the content of both the potential genotoxic impurities against the standard solutions prepared freshly. The data accessible in Table-Eight exposed that the solution was unchanging up to 24 hrs at room temperature.

TABLE 8: SOLUTION STABILITY DATA-FADACP, N NITROSO IMPURITIES AT 2.5PPM

Conditions	FADCP Content in PPM	N NITROSO Content in PPM
At 0 hrs	2.56	2.40
At RT for 24 hrs	2.54	2.40

CONCLUSION:

Accurate, sensitive, selective, specific analytical method developed for the quantification of impurities G and S in Valciclovir Hydrochloride, Hydrate API at 0.05% with respect to Valaciclovir Hydrochloride, Hydrate API sample concentartion using liquid chromatograph mass spectrometer. Electrospray ionization source/probe was used in positive mode of ionization. Also verified that LC-MS method is sensitive and effective than TLC method for the quantification of impurities G and S.

Specificity, precision, linearity, accuracy and solution stability studies was performed to validate the analytical method. The method specificity was proved by acceptable resolution of impurities with the Valaciclovir Hydrochloride Hydrate API. This method linearity covered in the range of 207.20 ppm to 777.00 ppm with respect to Valaciclovir Hydrochloride Hydrate API sample for impurity G, 216.00 ppm to 810.00 ppm with respect to Valaciclovir Hydrochloride Hydrate API sample for impurity S with a coefficient correlation of 0.9984 & 0.9976 respectively. The method accuracy was confirmed by the recovery values in the range of 96.0 % to 98.3 % for impurity G, 98.02 % to 103.4 % for impurity S with % RSD 1.53 & 2.96 respectively. This developed method is sensitive with a lower limit of quantification of 207.20 for impurity G and 216.00 ppm for impurity S.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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