GTIs evaluation: analytical tactic to quantitate the GTIs at the Threshold of Toxicological Concern level

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Abstract

The toxicity of Abacavir sulfate API impurities, N NITROSO [2-Amino-5-nitroso-4,6-pyrimidinediol] and FADCP [2-Amino-4,6-Dichloro-5-Formamido Pyrimidine] were checked by the software model Case Ultra and the study result was class 3 potential genotoxic impurities (GTIs). A delicate analysis method was developed for the impurity's quantification followed by the validation study, employing an electrospray ionization probe with a triple quadrupole liquid chromatography mass spectrometer.

Multiple reaction monitoring tactics were selected in positive ionization for the impurity's quantification of Abacavir sulfate. A gradient mode system was used by employing, 1.0ml of formic acid in water and organic solvent acetonitrile (LCMS grade) as a mobile phase A and B separately with SB Phenyl Zorbax (5.0µm, 4.6mm x 250mm) analytical column. The developed quantification method flow rate was set at 0.5 mL/min whereas 45 minutes was the run time. The developed method linearity range for FADCP started from 0.76ppm and ended at 3.79ppm. For N NITROSO, it started from 0.75ppm and ended at 3.76ppm when compared with the concentration of Abacavir sulfate drug, showing 1.0 and 0.9996 correlation coefficients separately. The method recovery was 111.7% to 98.7% for N NITROSO impurity and 101.7% to 104.3% for FADCP impurity.

Keywords: QSAR, Method Validation, Genotoxic.

Introduction

[(1S,4R)-4-[2-amino-6-(cyclopropylamino)purin-9-yl]cyclopent-2-en-1-yl]methanol;sulfuric acid is the organic name of the Abacavir sulfate, active pharmaceutical ingredient. It fits in NRTIs (Nucleoside reverse transcriptase inhibitors) class and is typically administered as either 300 mg dose two times or 600 mg dose one time daily to treat human immunodeficiency virus (HIV) infection in mixture with other medicines.

Scientific publications are available in public domain for the Abacavir sulfate genotoxic impurities (GTIs) quantification such as Impurity-II, Impurity-I, 4,6-Dichloropyrimidine-2,5-diamine and Impurity-III[3]. itionally, literature has discussed forced degradation studies, chromatographic analysis method development and quantitative evaluation of related substances in Abacavir Sulfate[4,6-8,15].

Some studies have focused on specific impurities like FADCP, but there remains a gap in methods for detecting N NITROSO and FADCP genotoxic impurities of Abacavir Sulfate API using LCMS-MS with a specified limit not exceeding 2.5ppm relative to the sample. The pharmaceutical companies manufactured APIs chemically or by doing modifications in natural processes. The process of manufacturing involves a variety of reagents, intermediates and catalysts. As a result of these chemical reactions, different impurities are formed which may originate from by-products of the reaction, reagents, or intermediates. The occurrence of impurities is a common occurrence in small quantities during the synthesis of various type of active pharmaceutical ingredients.

Impurities that arise while synthesizing can be detrimental, potentially showing genotoxic or carcinogenic traits. The impurities existence in API is a reason for worry among pharmaceutical companies and regulatory agencies. Genotoxic impurities possess the capability to engage with deoxyribonucleic acid (DNA) and present carcinogenic hazards to individuals. The detection of genotoxic impurities can be enhanced by employing toxicological software or by identifying particular functional groups present in the chemical structure. These potential genotoxic impurities are responsible for cancer in humans and are identified through structural alerts provided by toxicological software. 1.5 µg/day daily dose limit is recommended for potential genotoxic impurities by regulatory agencies to ensure patient safety.

Toxicological assessment and prediction of impurities structure utilizing Ultra Case Software: The evaluation of the toxicological potential and safety limits of impurities was conducted using Quantitative Structural Activity Relationship (QSAR) analysis in line with the (ICH) International Conference on Harmonization guideline M7 (R2). DNA-reactive (mutagenic) impurities valuation and management are well described to mitigate the risks related to carcinogenicity. A toxicology assessment is required to evaluate the assay of bacterial mutagenicity, employing two distinct tactics, one is expert rule-based and the other one is statistical-based, both integral to (Q)SAR models. Furthermore, (Q)SAR methodologies and computational toxicological prediction validation principles have been delineated in the OECD (Organization for Economic Co-operation and Development) guidelines, which are essential for toxicological assessments.

The toxicological evaluation of N NITROSO impurity and FDACP impurity is performed using the Case ultra-

software, a predictive toxicology program that integrates both expert rule-based and statistical methodologies. This software employs a knowledge-based data base prearrangement so that it incorporates rules of expert to provide qualitative risk estimations. The risk is categorized qualitatively as 'certain', 'probable', 'plausible', 'equivocal', 'doubted', 'improbable' and 'impossible'. *In vitro* mutagenicity of bacterial negative prediction permits an 'inactive' classification, while compounds which are non-alerting, are further analyzed and are separated into misclassified and unclassified characteristics.

The strategy which is based on rule, entails the evaluation of different fragments related to molecule to comprehend the variations in biological response according to established chemical guidelines. Moreover, it considers cross-term fragment descriptors to pinpoint essential fragment interactions affecting activity diversity. On the other hand, the sophisticated technique employs models related to QSAR for statistical assessment and to offer a predictive approximation of chemical toxicity quantitatively. This algorithmic *in silico* framework depends on distinct mutagenicity structural alerts found in the literature. The projected mutagenesis likelihood is tailored to the input structure sub-model.

Figure 1 illustrates the chemical structures of Abacavir Sulfate, FADCP and N NITROSO impurities. FADCP and N NITROSO impurities toxicological summary are outlined in table 1. Abacavir sulfate API's maximum daily dose is 600 mg in a day. TTC (Threshold of Toxicological Concern) calculation is directly proportional to the daily dosage of the product and according to the calculation, in Abacavir sulfate API, N NITROSO and FADCP levels should not surpass 2.50ppm. Hence, it is crucial to regulate both the genotoxic impurities as per the level 2.50ppm concerning the concentration of the Abacavir sulfate API drug. Analytical instruments having lower concentration detection ability are essential to control the genotoxic impurities.

There are some techniques widely used in the industry, especially in pharmaceutical field, like UV spectrophotometry, GC and HPLC which are inadequate for detecting genotoxic impurities at low concentrations because of their restricted detection at lower level. Improved lower-level detection can be attained through the employment of advanced analytical instruments such as LC-MS/MS, GCMS etc.

Many researchers have favored the MS technique for quantifying impurities at a very low level because of the consistency of the LCMS-MS analysis technique. Therefore, considering the 2.50ppm threshold for quantifying both potential genotoxic impurities concerning the concentration of the Abacavir sulfate drug API sample, a method was devised utilizing an LC-MS/MS technique.

Contaminants Source: In the Abacavir sulfate route of synthesis, sodium hydroxide solution is used with an intermediate FADCP. The hydrolysis reaction of FADCP under basic conditions takes place and forms N NITROSO impurity during the synthesis process.

Material and Methods

Mass spectrometry grade, Bioslove make, acetonitrile organic solvent and formic acid were arranged for the study. Water system of Milli Q -Millipore was used to get purified water.

Table 1
Toxicological Summary

Impurity	Genotoxic Predictions considering Bacterial Mutagenicity Model of OECD 471	Genotoxic Predictions for Bacterial Mutagenicity Model as per Expert Rules	Classification
FADCP	Result: Positive	Result: Positive	Mutagenic- Class 3
	Possibility: 62.1%	Possibility: 75.9 %	
N NITROSO	Result: Positive	Result: Positive	Mutagenic- Class 3
	Possibility: 86.6%	Possibility: 94.9 %	

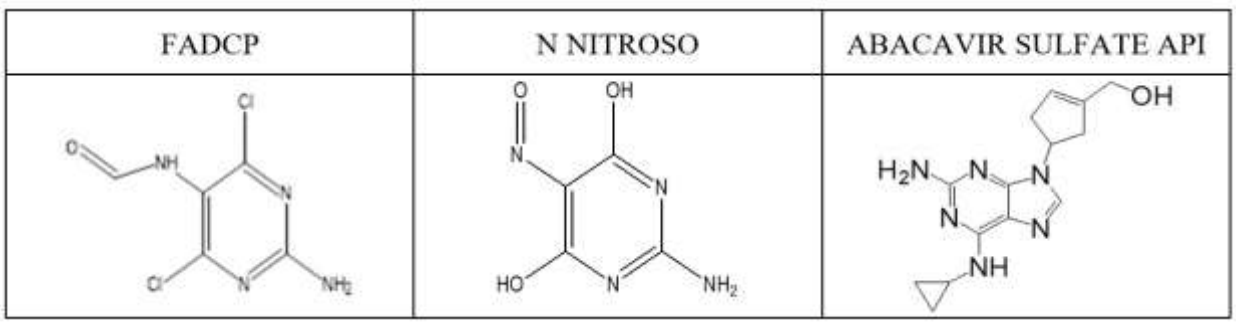

Figure 1: Structural Details

The Abacavir sulfate active pharmaceutical ingredient (API) sample was graciously supplied as a complimentary sample by the manufacturer whereas the potent impurities were sourced from the impurities manufacturer available in India. Additionally, 25% solution of ammonia was procured from Merck.

Equipment: The LC-MS/MS system employed in the development and validation of potential genotoxic impurities was the AB Sciex 5500 model- Applied Biosystems, from Singapore. This model was combined with Germany manufactured LC components including a Binary pump 1290 Agilent, a DAD detector 1290 Agilent, an Infinity sampler 1290 Agilent and an Infinity column thermostat 1290 Agilent. Software Analyst 1.6.2 version was used for data generation and further processed on a Dell computer. The Zorbax SB phenyl 250 mm analytical LC column with 5.0 µm x 4.6 mm dimension from Agilent Technologies, was utilized for this purpose.

The mobile phases utilized in this study included phase A which comprised the mixture of formic acid (0.1%) in water and phase B, consisting of organic solvent acetonitrile (v/v), operated in a gradient system. 0.50 mL per minute flow proportion of the mobile phase was established with a flow splitter reducing the flow to 0.30 mL per minute fixed into the mass spectrometer. The 55°C temperature was maintained for the column oven, while 5°C temperature was set as a cooler temperature. A 10 µL injection volume was employed for the study. An electrospray ionization source as a probe was utilized to identify the Abacavir sulfate, potential genotoxic impurities. Using a positive ionization approach, the mass spectrometry method implemented multiple reaction monitoring (MRM).

Identified FADCP and N NITROSO impurities are in positive mode of ionization with MRM transition m/z [207 - 171] and m/z [157- 96] separately. For the FADCP impurity, set 120 V declustering potential and 6 V entrance potential, while for the N NITROSO impurity, set 50 V declustering potential and the 10 V entrance potential. 5500 V was kept as an ion spray voltage and 30 psi and 60 psi pressures were set for the nebulization for ion source as gases 1 and 2 respectively. This developed LC-MS/MS method demonstrates efficacy in quantifying both genotoxic impurities present in the active pharmaceutical ingredient Abacavir sulfate.

Preparation of Solutions for Standard and Sample Analysis: Diluent (Water and acetonitrile mixture in 50:50 v/v composition) was used to prepare the 0.05 mg per mL FADCP impurity solution. N NITROSO impurity, 10 ml standard solution was organized containing 0.05 mg per mL concentration solution in ammonia solution (25% V/V) of 0.1% and then filled up to the mark with diluent. 0.50 mL of impurity solution was prepared in a 50 mL container glass flask and additional filled up to desired volume with the diluent. Consequently, 0.0005 mg/mL, a weakened stock solution of N NITROSO and FADCP impurities was formulated.

The ultimate standard solution was created in 10 mL capacity container by diluting 0.50 mL of the diluted weakened stock solution and filled with diluent up to the desired volume, resulting in a concentration of 2.50ppm in relation to the Abacavir sulfate concentration. Abacavir sulfate sample solution was prepared as 10 mg in one mL meditation in the diluent by melting a suitable quantity. Prepare impurity solutions for FADCP and N NITROSO in ppm 0.75, 2.50 and 3.75 concentration for the recovery studies whereas solutions were also prepared at 0.75, 1.25, 2.50, 3.00 and 3.75ppm for the linearity study. Before analysis, all standard solutions prepared underwent sonication.

Analytical approach for method development: The current strategy involved the examination of various types of analytical column stationary phases to achieve a significant separation between genotoxic impurities and the Abacavir sulfate drug substance. The structures of genotoxic impurities closely resemble those of Abacavir Sulfate drug.

To accomplish the necessary detection and resolution among Abacavir sulfate and their impurities, as well as the accuracy as per the acceptable limit, several columns such as C18 Waters, C18 Ascentis Express, YMC Triart EXRS C18, SB Phenyl Zorbax with different column dimension were estimated during development activity using different types of buffers as a mobile phases A and their combinations. All peaks were early eluted at the void volume when the mobile phase used 0.02% ammonia of 25% solution. The YMC Triart column was tested with the same mobile phase, but the genotoxic impurities response observed was lower.

Various mobile phase polarities were employed with different compositions for the development of the analytical method. Initially, an isocratic analysis method was tried, but finally, a gradient method was approved. Ultimately, a favorable resolution, intensity and recovery were achieved on the Phenyl Zorbax SB column (5.0µm,250 mm × 4.6mm) using mobile phase A consisting of 0.1% formic acid and mobile phase B of organic solvent acetonitrile. The gradient program established in line with the table 2, was successful in achieving the desired resolution between both the genotoxic impurities and Abacavir Sulfate peak. Set column thermostat temperature was 55°C and between 20 minutes to 45 minutes retention time, the diverted valve was used successfully to avoid any additional interference in the mass spectrometer instrument.

Results and Discussion

Validation-Analytical Method: The specificity of the method was validated through the analysis of the Abacavir sulfate sample solution, blank solution, impurities solution of each genotoxic impurity separately. Figures 2-5 displayed the chromatograms which illustrated the absence of

interference at the elution periods of the Abacavir sulfate medication and the possible genotoxic contaminants.

FADCP and N NITROSO impurities chromatograms revealed that FADCP impurity eluted at 16.48 min and N NITROSO impurity eluted at 7.0 min respectively. The LC-MS/MS developed method successfully separated the genotoxic impurities from one another. Genotoxic impurities standard solutions with lower concentrations were prepared to establish the lower limit of quantification, resulting in S/N ratios of 40 (Figure 6) for FADCP and 69.7 (Figure 7) for N NITROSO impurities. Both impurities had a LOQ of 0.75 ppm, with a LOD of 0.25 ppm. The findings regarding precision level LLOQ data are summarized in table 6 which details the data for the LLOQ, limit level and 150% level. Determine the method linearity with varying concentrations of potential genotoxic impurities by selecting multiple reaction monitoring tactic, ranging from 0.75 ppm to 150% of the limit level concentration (3.75 ppm). A calibration curve was generated by plotting peak areas against impurities concentrations (0.75, 1.25, 2.50, 3.00 and 3.75 ppm). From the regression analysis, the correlation coefficient, slope and intercept values obtained are briefed in table 3.

The precision of the method was assessed at 100%,150% and LLOQ level of the target concentrations through a spiking activity involving potential genotoxic impurities, specifically 0.75, 2.50 and 3.75 ppm in relation to the concentration of Abacavir sulfate. The findings regarding accuracy are summarized in table 4, which details the data for the LLOQ, limit level and 150% level.

It is expected that the potential genotoxic impurities accuracy percent relative standard deviation not to exceed 10.0% with an accuracy range of 85.0% to 115.0% at these specified levels.

Table 2
Gradient Details

Time (minutes)	Mobile phase A %	Mobile phase B %
0.01	94	6
18	94	6
23	12	88
29	12	88
33	94	6
45	94	6

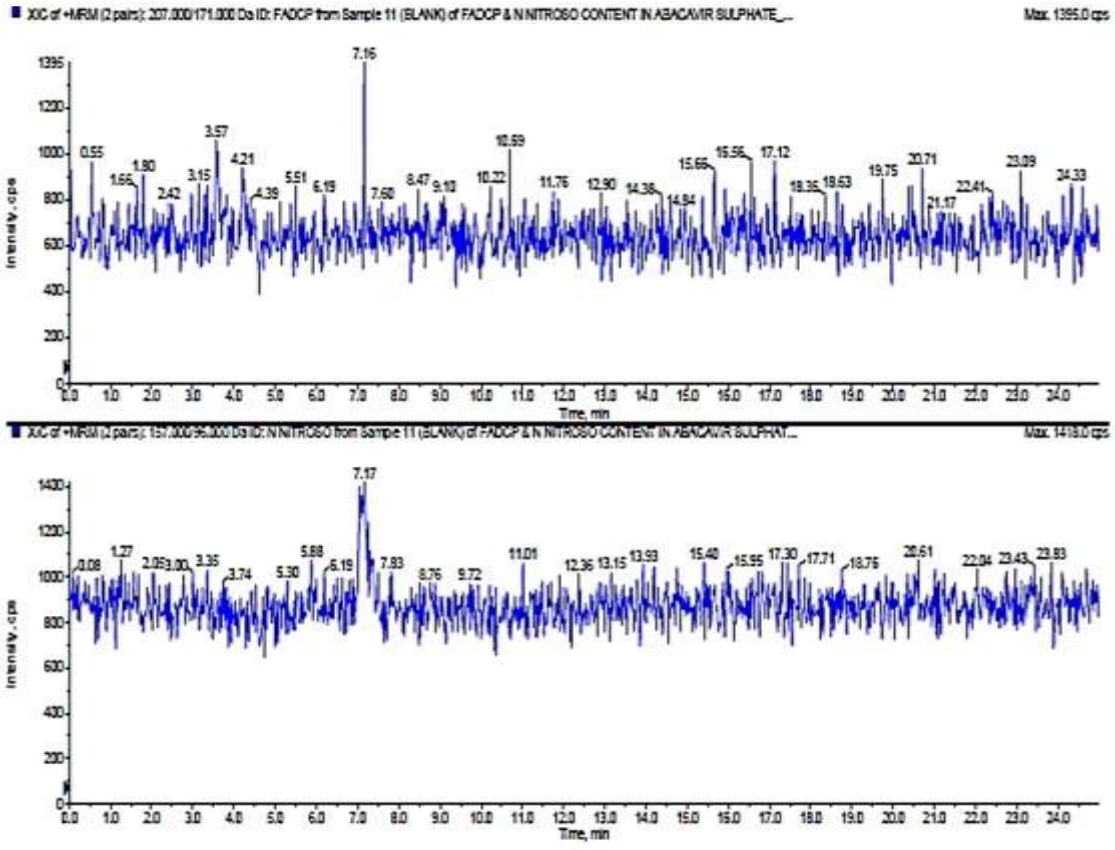

Figure 2: Blank Chromatogram

Table 3
Linearity

Linearity Levels	Impurity- FADCP Concentration in ppm	Area- FADCP	Impurity- N NITROSO Concentration in ppm	Area- N NITROSO
Level-1, 30%	0.76	174636	0.75	86680
Level-2, 50%	1.26	286697	1.25	140599
Level-3, 100%	2.53	563917	2.50	275547
Level-4, 120%	3.03	679310	3.00	325180
Level-5, 150%	3.79	845590	3.76	399573
Correlation Coefficient(r^2)		1.000	Correlation Coefficient(r^2)	0.999
Slope		221291	Slope	104596
Intercept		6694	Intercept	9860

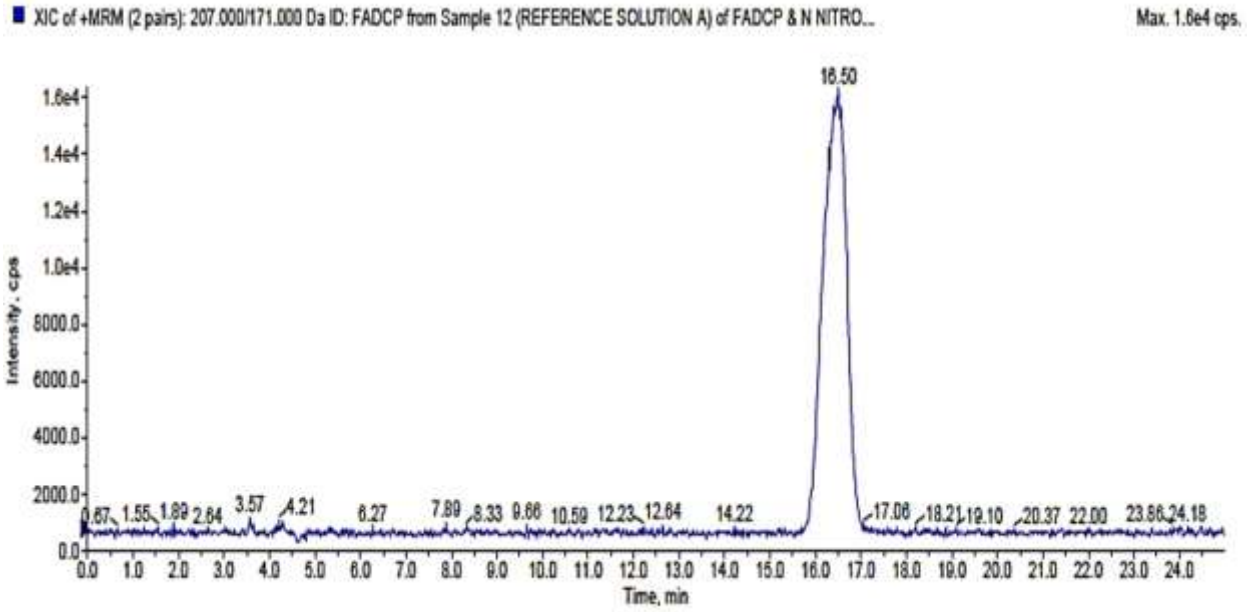

Figure 3: Extracted ion chromatogram – FADCP

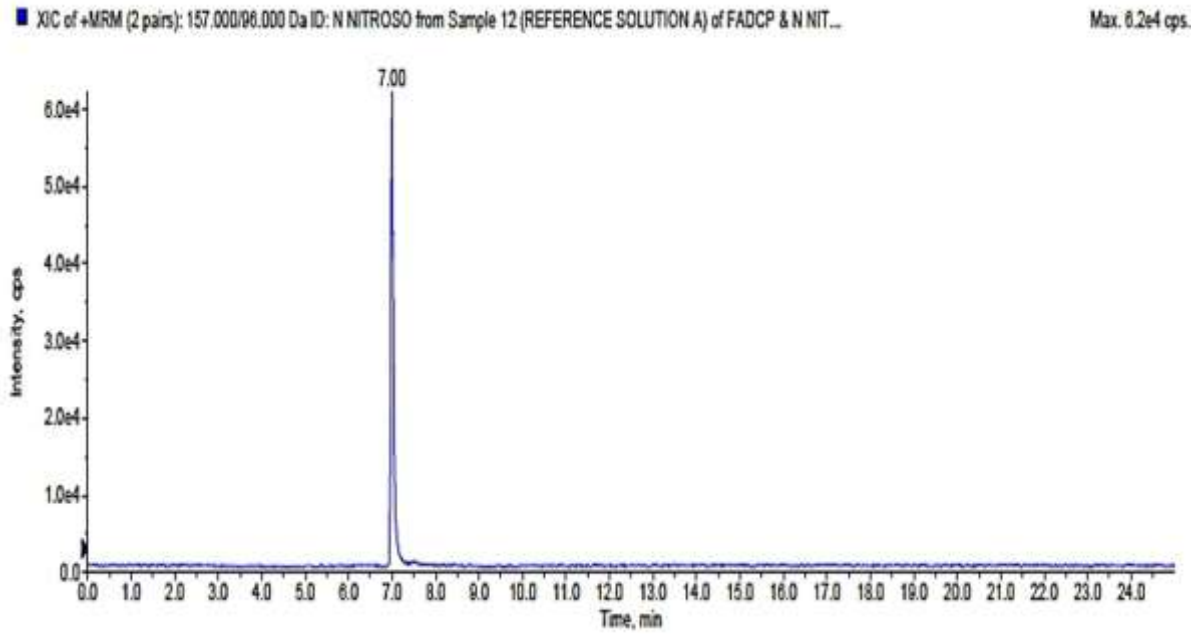

Figure 4: Extracted ion chromatogram - N NITROSO

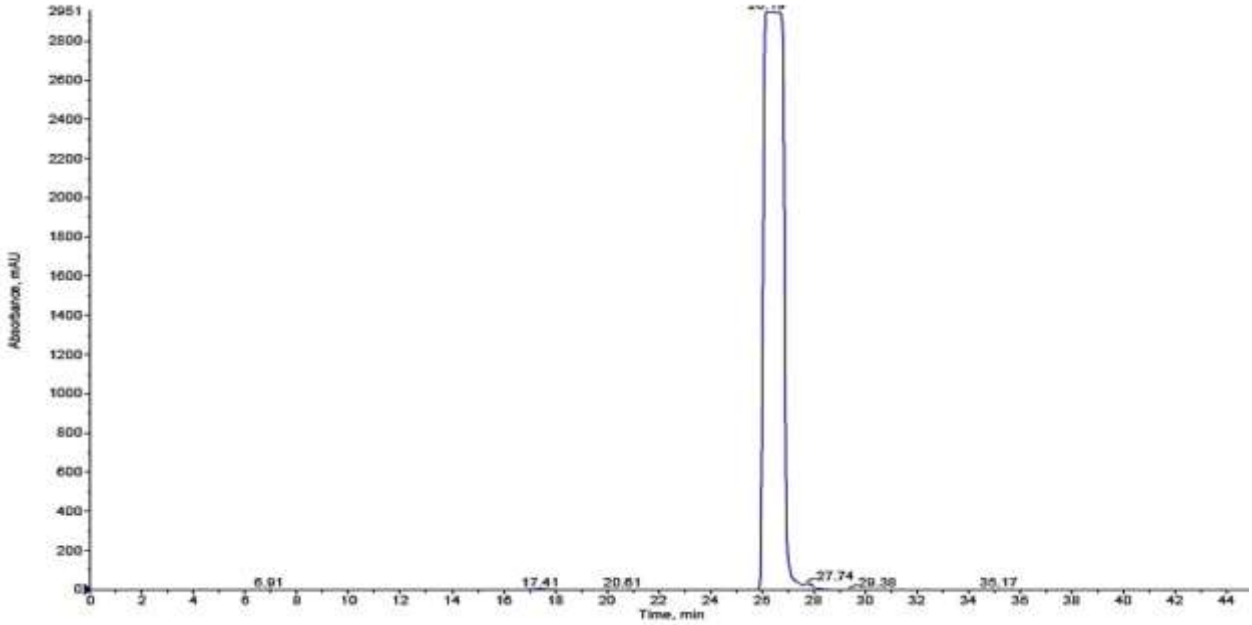

Figure 5: Abacavir Sulfate Chromatogram

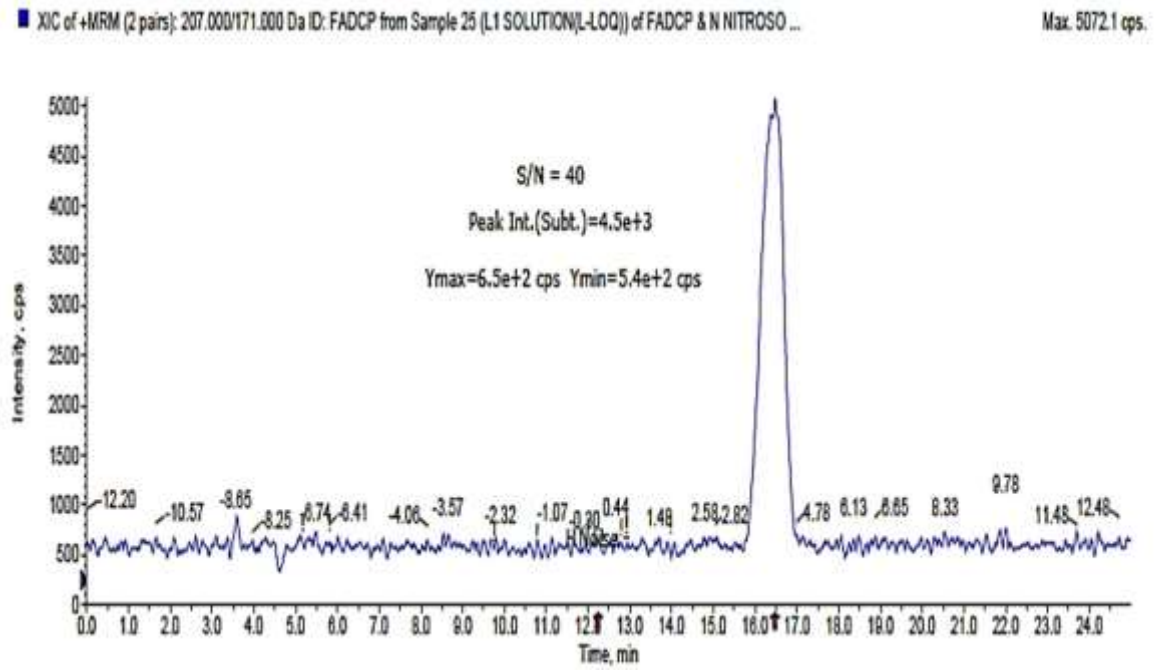

Figure 6: S/N ratio- FADCP

Table 4
Accuracy

Impurity Name	**FADCP Concertation in ppm**			**N NITROSO Concertation in ppm**		
	Theoretical	Obtained	Accuracy %	Theoretical	Obtained	Accuracy %
Accuracy Level 1-LLOQ 30%	0.761	0.772	101.73	0.752	0.842	111.74
Accuracy Level 2-100% Level	2.531	2.562	101.34	2.505	2.524	100.63
Accuracy Level 3-150% Level	3.792	3.953	104.33	3.766	3.703	98.72
Relative Standard Deviation %			**1.62**	**Relative Standard Deviation %**		**6.77**

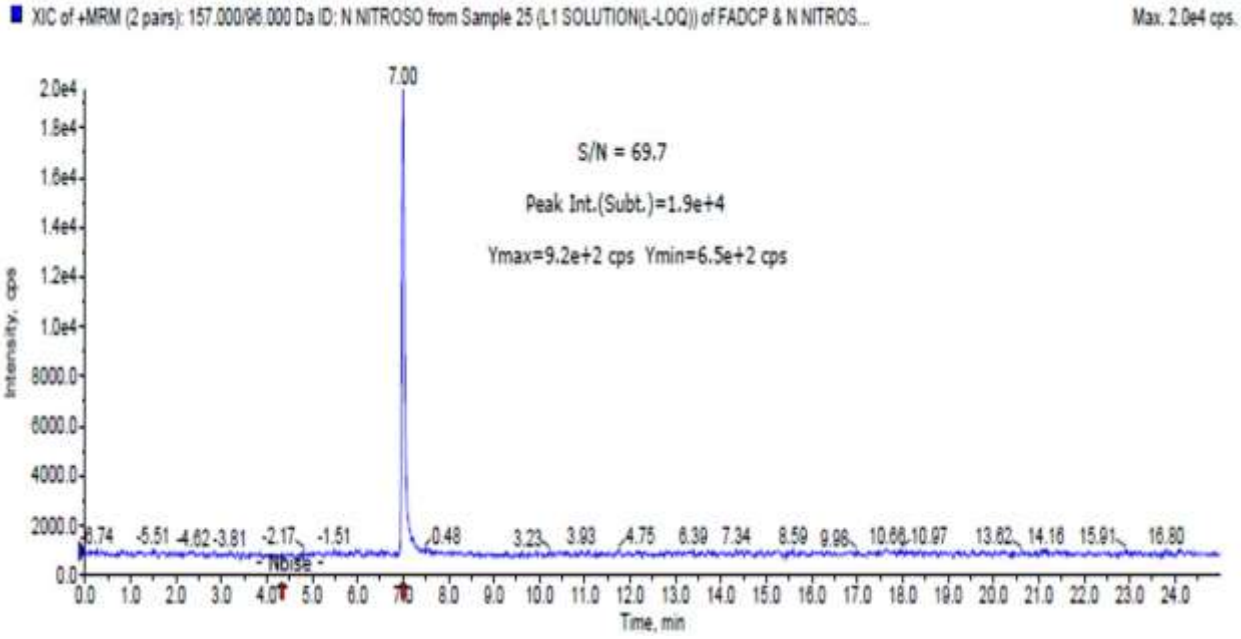

Figure 7: S/N ratio- N NITROSO

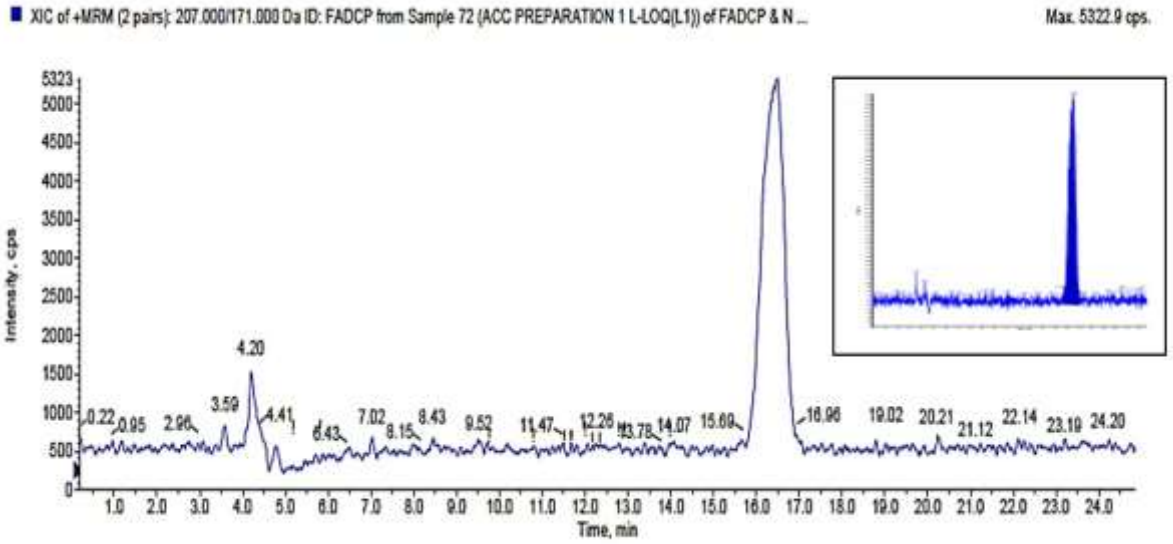

Figure 8: XIC LLOQ Spiked Sample- FADCP

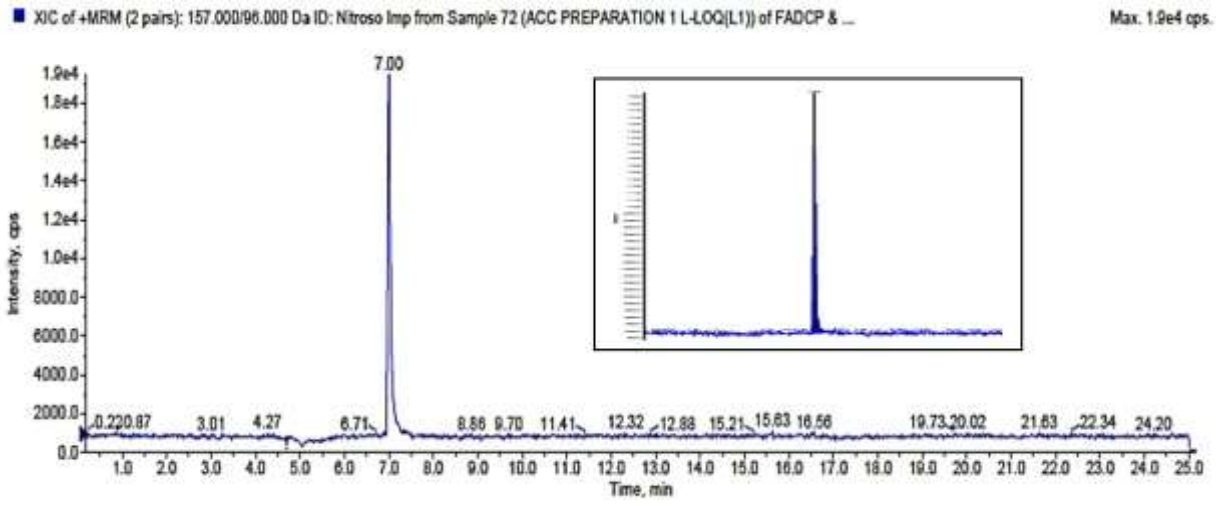

Figure 9: XIC - LLOQ spiked sample - N NITROSO

The accuracy study result for the FADCP impurity was found to be between 101.73% and 104.33%, with an RSD of 1.61%, while for the N NITROSO impurity, the recovery values ranged from 111.68% to 98.62%, with an RSD of 6.76%. Figure 8 and figure 9 indicate spiked extracted ion chromatograms of impurities FADCP and N NITROSO at LLOQ level concentration. The study related to repeatability and ruggedness was done to determine the method's precision.

Using the spiking study tactic, the repeatability of the method was assessed. Spike the standard concentration at specification level into six sample solutions which were freshly prepared on the same day and each impurity content relative standard deviation was evaluated. The results, which are available in table 5, indicate that for N NITROSO and FADCP impurities, the relative standard deviation was 1.77% and 0.93% respectively. Additionally, six replicate injections of LLOQ level were done to evaluate the precision at the LLOQ level with the % relative standard deviation being 2.16% and 2.31 % for N NITROSO and FADCP impurities separately as per table 6. The recovery percentages for the FADCP impurity ranged from 101.73% to 104.33%, with a relative standard deviation (RSD) of 1.61%.

On the other hand, the N NITROSO impurity exhibited recovery values between 111.68% and 98.62% with an RSD of 6.76%. The ruggedness of the method was evaluated by examining the levels of both the impurities in sample solutions which were prepared freshly on separate days, following specific standard criteria. The total impurity content relative standard deviation between the spike precision and intermediate precision must not exceed 10.0% as in table 7. The method robustness parameter was studied by constructing deliberate changes in the flow rate of mobile phase and column thermostat temperature.

Flow rates of the mobile phase along with the column temperature variations effects were studied. Specifically, the flow rate of the developed method (0.50 mL/Min) was modified by 10%, ranging from 0.45 mL per minute to 0.55 mL per minute, while the thermostat column temperature was changed from 55ºC to 53ºC and 57ºC. The modifications made during the robustness assessment did not lead to significant alterations in the resolution of impurities from Abacavir sulfate in the analysis. To establish the analytical solution's stability, impurity solutions at target levels spiked with Abacavir sulfate drug concentration were stored for 24 hours at room temperature (25ºC). Compare the levels of potential genotoxic impurities with freshly prepared impurities standard solutions and evaluate the stability of sample solutions. The analytical summary presented in table 8 demonstrated that the sample solution exhibited no changes for a period of 24 hours when stored at room temperature.

Table 5
Spike Precision

Injection Details	FADCP Impurity content found in sample	N NITROSO Impurity content found in sample
Injection-1	2.51	2.50
Injection-2	2.48	2.46
Injection-3	2.48	2.48
Injection-4	2.51	2.44
Injection-5	2.49	2.45
Injection-6	2.53	2.41
Mean Content	2.50	2.44
Standard Deviation	0.02	0.04
Relative Standard Deviation %	**0.94**	**1.78**

Table 6
Precision at LLOQ Level

Injection Details	FADCP Impurity Standard solution area in LLOQ Solution	N NITROSO Impurity Standard solution area in LLOQ Solution
Injection-1	175364	84625
Injection-2	181567	87176
Injection-3	169253	84953
Injection-4	175033	85742
Injection-5	173337	88438
Injection-6	173262	89147
Mean Area	174636	86680
Standard Deviation	4031.8	1871.3
Relative Standard Deviation %	**2.31**	**2.16**

Table 7
Ruggedness

Injection Details	FADCP Impurity Concentration obtained in sample	N NITROSO Impurity Concentration obtained in sample
Precision-1	2.51	2.50
Precision-2	2.48	2.46
Precision-3	2.48	2.48
Precision-4	2.51	2.44
Precision-5	2.49	2.45
Precision-6	2.53	2.41
Ruguddness-1	2.69	2.67
Ruguddness-2	2.56	2.65
Ruguddness-3	2.69	2.71
Ruguddness-4	2.76	2.68
Ruguddness-5	2.76	2.70
Ruguddness-6	2.68	2.53
Mean	**2.60**	**2.55**
Standard Deviation	**0.106**	**0.131**
% R.S.D.	**4.14**	**5.13**

Table 8
Solution stability

Situations	FADCP impurity found in PPM	N NITROSO impurity found in PPM
At 0 hrs.	2.57	2.41
At RT for 24 hrs.	2.53	2.39

Conclusion

Using an ESI (electrospray ionization) probe with a mass spectrometer, we developed a precise and novel analysis method to quantify genotoxic impurities FADCP and N NITROSO at a concentration of 2.5ppm relative to the Abacavir Sulfate drug level. The validation of this approach involved evaluating all validation parameters like linearity, accuracy, precision, solution stability and specificity. The method's specificity was ascertained through effectively separating impurities from the Abacavir sulfate drug. The linearity range extended from 0.76ppm to 3.79ppm for FADCP impurity whereas for N NITROSO impurity it was 0.75ppm to 3.76ppm in relation to the Abacavir sulfate drug concentration, with correlation coefficients of 1.00 and 0.9996 respectively.

The method accuracy recovery values ranging from 101.7% to 104.3% for FADCP impurity and 111.7% to 98.7% for N NITROSO impurity confirmed the accuracy of the method. The % relative standard deviation values were found 1.61 and 6.76 respectively in the accuracy study. This method developed is effective, to quantitate 0.75ppm for potential genotoxic impurities as a lower limit of quantification (LLOQ).

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(Received 04th August 2024, accepted 24th September 2024)