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Original Research article

A Simple and Sensitive Method for the Ultra Trace Determination of Potential Genotoxic Impurities in Abacavir Sulfate by LC-MS

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ABSTRACT

A new LC-MS based method was developed to detect three potential genotoxic impurities namely, N',N'''-(4,6-dichloropyrimidine-2,5-diyl)bis[N,N-dimethyl(imidoformamide) (Impurity-I), {N-(2-amino-4-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), at low level in Abacavir sulphate as a nucleoside reverse transcriptase inhibitor. It utilizes zorbax phenyl hexyl column (150 mm x 4.6 mm, 3.5 µm) with electrospray ionization in selected ion monitoring (SIM) mode for quantitation of these PGIs. The method is able to quantify Impurity-I at 0.74 ppm, Impurity-II and Impurity-III at 0.73 ppm with respect to 5.0 mg/mL of Abacavir sulfate. The method was found to be linear in the range of LOQ to 150% of Toxicological Threshold Concentration level of 2.5 ppm. The correlation coefficients of PGI's obtained were >0.999 in each case. The method accuracy of these PGI's is in the range between 88.7-115.0%. The method is sensitive, specific, linear, accurate, precise and meets the criteria of validation as per International Conference on Harmonization contends.

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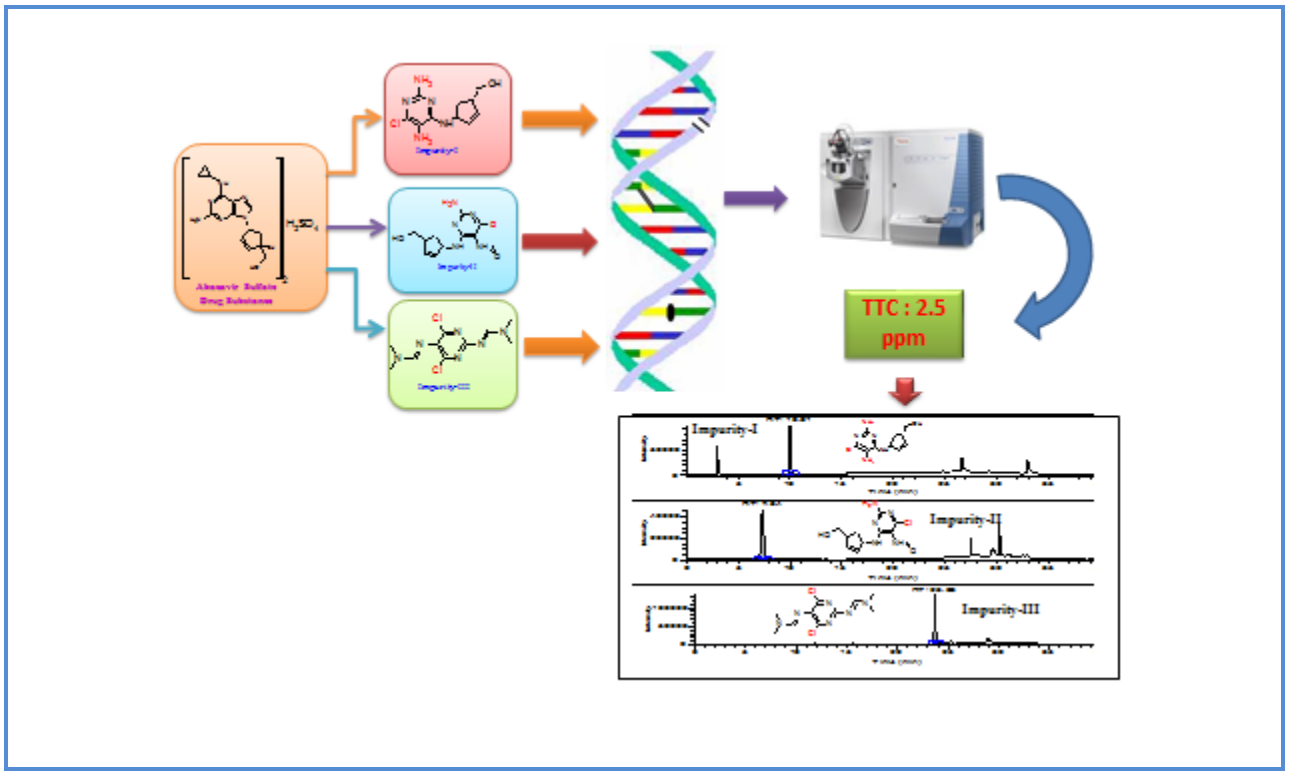
Graphical Abstract



Introduction

Over the last several years, various global regulatory agencies have been raising their concern to control the level of genotoxic impurities in active pharmaceutical ingredients [1-2]. Compounds, categorized as PGI's, actually include a broad range of unrelated chemicals with very different structures and from very different chemical families. If an impurity contains structural alerting functional group for mutagenicity and can react with our genetic material such as deoxyribonucleic acid (DNA), it can also be classified as a potential genotoxic impurity (PGI's) [3]. The European Medicines Agency and ICH has prescribed guidelines on the control of PGI's [3, 4] and accordingly PGI's must be controlled to a level below the TTC of 1.5 µg/day unless specific toxicological thresholds are established.

Abacavir sulfate, (1S, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9yl]-2-cyclopentene-1-methanol sulphate, is a synthetically prepared carbocyclic nucleoside analog reverse transcriptase inhibitor (NRTI) used to treat HIV and AIDS [5]. It is used either as a 600-mg once-daily or 300-mg twice-daily regimen [6]. It has molecular formula of $(C14H16N6O)_2$. H_2SO_4 and molecular weight 670.74 Da. The Abacavir sulfate formulation product is traded in the name of Ziagen as oral administration by innovator. It is listed in the United States pharmacopeia as well as European

pharmacopeia. Impurity-I, Impurity-II and Impurity-III are intermediates formed during the synthesis of Abacavir sulfate, from 2, 5-diaminopyrimidine-4,6-diol. These impurities are identified as PGI's based on the presence of functional groups with structural alert [7-9]. Chemical name, structure and molecular mass of Abacavir sulfate and its PGI's, are shown in [Table 1].

Based on the maximum daily dosage of abacavir sulphate, the TTC limit of PGI's was found to be 2.5 ppm. It requires high sensitivity of any method to detect at such a low level. On extensive literature search, few articles were found dealing with the identification and characterization the genotoxic degradation products of the Abacavir sulfate and development and validation of a reverse-phase liquid chromatographic method for assay-related substances of Abacavir sulfate [10-12] was found. However, none of these published literatures endorses any method for the determination of these three PGI's at TTC level. In the present study, we developed a LC-MS method and validated it as per ICH guidelines [13] for the determination of three PGI's in abacavir drug substance.

Experimental

Chemicals and reagents

The Abacavir sulfate drug substance, Impurity-I, Impurity-II, and Impurity-III were obtained from Micro Labs Limited (R&D), Bangalore, India. Ammonium acetate (AR grade) and formic acid (LC-MS grade) were obtained from fisher scientific (Mumbai, India), high purity acetonitrile and methanol (gradient grade) were obtained from Merck Life Sciences (Mumbai, India). Milli-Q water was prepared in-house by Milli-Q water purification system obtained from Merck Millipore (Bedford, MA, USA).

Instrumentation

LC-MS analysis was carried out on LTQ Velos Pro dual pressure linear ion tarp mass spectrometer (Thermo fisher, San Jose, USA) connected with a high pressure liquid chromatograph Nexera X2 (Shimadzu Corporation, Kyoto, Japan). MS-data was acquired automatically in positive ionization mode with 4.0kv ionization potential with a heated electrospray ionization (HESI) probe using Thermo Xcalibur software (Thermo Fisher Scientific, San Jose, USA). A single ion monitoring (SIM) scan was performed with a window of 256.0±1Da for ABA-1 Impurity-A, 284.1±1Da for ABA-1 and 289.0±1Da for ABA-S1 Impurity-D. The source temperature and capillary temperature were maintained at 350 ˚C. The sheath gas and auxiliary gas pressure were kept at 40 psi and 10 psi respectively.

Chromatographic conditions

The method was carried out using Zorbax phenyl hexyl C18, 150 mm length, 4.6 mm id and 3.5 micron particles size (Agilent Technologies, CA, USA). The eluent was a 10 mm ammonium acetate and acetonitrile in gradient mode (T_{min} A:B): $T_0$90:10, $T_3$90:10, T_{20}75:25, T_{25}20:80, T_{30}10:90, T_{31}90:10, T_{40}90:10. The flow rate, column oven temperature and auto sampler temperature were set as 0.7 mL/min, 40 ^{o}C and 5 ^{o}C respectively. The injection volume was set as 10 µL. The flow of eluent was diverted to waste from 13 min. to 16 min. in order to reduce source contamination.

Sample and standard preparation

Sample solutions of ABA were prepared at 5000 ppm in diluent (0.1% formic acid in methanol). The standard solutions of PGI's were prepared at concentration of 2.5 ppm with respect to ABA in diluent for system suitability. The concentration of the standard solutions and samples were optimized to achieve a required LOD, LOQ and good peak shape. All the standards were well-sonicated and, then, filtered through 0.22 µm membrane filters prior to their analysis.

Results and discussion

Optimization of diluent (sample preparation)

Different diluents were evaluated with respect to chromatographic efficiency of PGI's. Solution stability and recovery of the PGI's solution were found good in 0.1% formic acid in methanol. The symmetrical peak shapes were obtained in 0.1% formic acid in methanol. Addition of 0.1% formic acid, not only improved peak shape, but also helped getting recoveries. Accuracy in the range 88-115% was also achieved for PGI's in 0.1% formic acid in methanol.

Optimization of chromatographic condition

The preliminary aim was to separate the PGI's from each other, along with ABA peak, due to their similar chemical structure and polarities. As the PGI's were having low level of specification limit (2.5 ppm), we found that the HPLC method did not exhibit the desired sensitivity to these PGI's. Hence, an LC-MS was developed to detect the PGI's at the level of toxicological threshold of concern (TTC) level. The PGI's Impurity-I, Impurity-II and Impurity-III, with the protonated masses as m/z 286 Da, 284 Da and 289 Da respectively, were optimized for their response under different trials on changing the voltages and temperatures in selective ion monitoring (SIM) mode. The chromatographic conditions were described in section 2.3. Several trials were conducted in different types of commercially available columns namely Inertsil ODS 3V (150 mm x 4.6) mm, 5 µm, Kromasil C8(150 mm x 4.6) mm, 5 µm, Zorbax SB phenyl (150 mm x 4.6) mm, 5 µm and Zorbax

phenyl-hexyl (150 mm x 4.6) mm, 3.5 µm. These columns were evaluated based on their stationary phase, packaging material, particle size and their ability to cause separation among all the analytes of the interest. The Inertsil ODS 3V, Kromasil C8 and Zorbax SB phenyl were not effective due to poor separation amongst impurities and the drug substance peaks. Zorbax phenyl-hexyl column produces a clear separation among all the PGI's and ABA with improved peaks shape. Different compositions of mobile phase using volatile buffer such as formic acid, ammonium acetate, acetonitrile-methanol (90:10, v/v), acetonitrile-methanol (70:30, v/v), methanol and acetonitrile were studied. However, appropriate separation and peak shape was with acetonitrile and 5 mm ammonium acetate. Isocratic as well as gradient elution modes were evaluated and gradient elution was found to be more efficient in achieving optimum separation amongst analytes and drug substance peak.

Method Validation

The proposed method was validated as per ICH guidelines [10] in terms of specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, robustness and solution stability. The LOD and LOQ for all three PGI's were determined by injecting diluted solutions with known concentrations as shown in [Figure 1 (a-f)]. Precision study was also carried out at the LOQ level by injecting six replicate, at predicted concentration, and calculating their % RSD value. Linearity of the method was evaluated at six concentration levels from LOQ to 150% of impurity concentrations (0.73, 1.25, 2.0, 2.50, 3.0 and 3.75 ppm for all PGI's). The linearity was satisfactorily demonstrated with a six point calibration graph between LOQ and 150% of the impurities concentration. The slope, intercept and regression coefficient values were determined by the least squares linear regression analysis. The accuracy of the method was evaluated in triplicate using three concentration levels at LOQ, 2.5 ppm and 3.75 ppm i.e. (LOQ – 150% of specification level). The % recovery and % RSD for all three PGI's were calculated. The precision of the method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual preparations of abacavir sulfate sample solution spiked with impurities at 100% concentrations. The % RSD of area for each individual impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and performing the analysis on different days and different analyst. Solution stability of the impurities in sample solution was established by analyzing spiked sample solution at different time intervals at 5°C. The robustness of the method was studied with deliberate alteration in the flow rate of the mobile phase and column temperature. The optimized flow rate of the mobile phase was 0.7 mL/min and the same was

altered by ±0.2 units i.e. from 0.5 mL/min to 0.9 mL/min. The effect of column oven temperature was studied at 38°C and 42°C instead of 40°C. The optimized ionization energy was 4.0Kv and same was altered by ±0.5 units i.e. from 3.5Kv to 4.5Kv. The optimized sheath gas and auxiliary gas pressure at 40 psi and 10 psi respectively and the same were altered by ±5.0 units i.e. from 35 psi to 45 psi and 5 psi to 15 psi respectively. Method validation results are summarized in [Table 2-3].

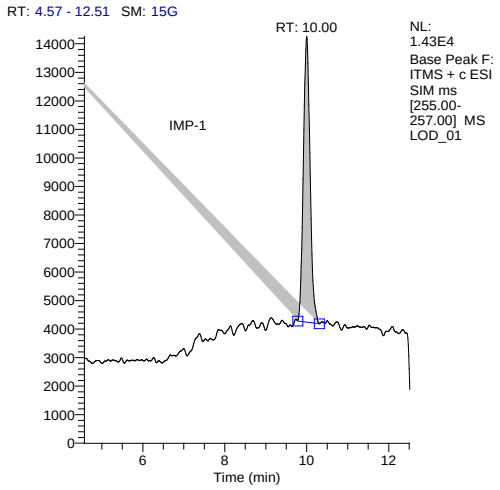


Figure 1a. Typical Chromatogram of Impurity-1 at LOD level

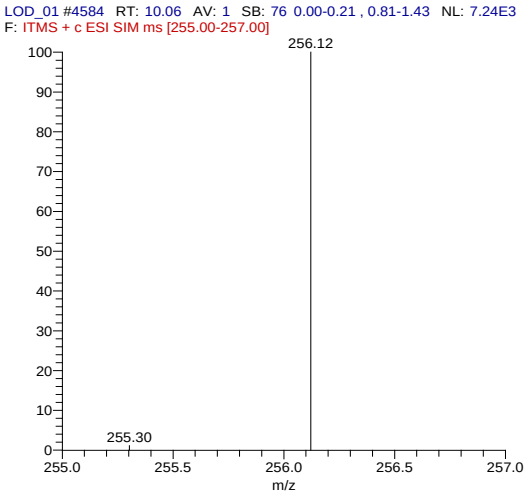


Figure 1b. Mass spectrum of Impurity-1 at LOD level

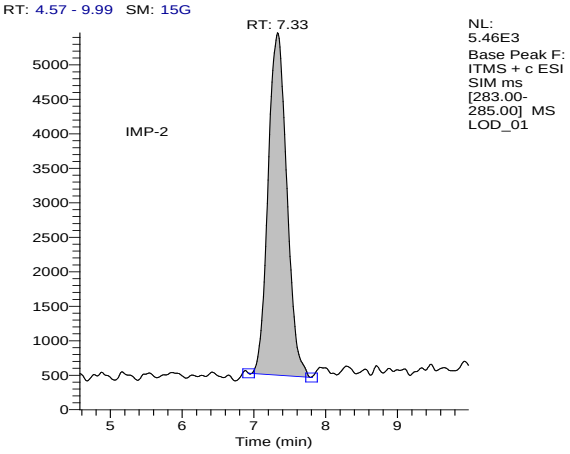


Figure 1c. Typical Chromatogram of Impurity-2 at LOD level

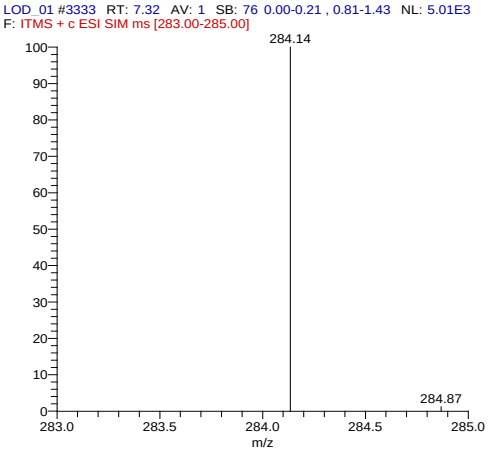


Figure1d. Mass spectrum of Impurity-2 at LOD level

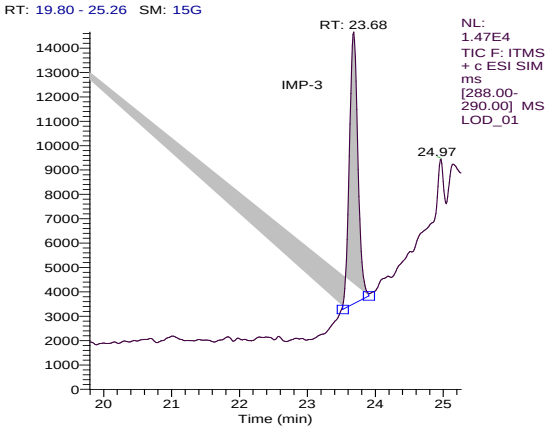


Figure 1e. Typical Chromatogram of Impurity-3 at LOD level

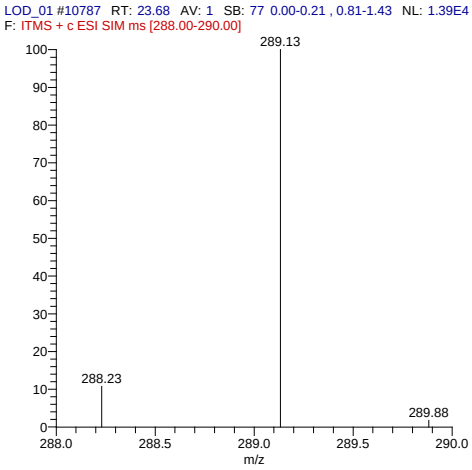


Figure 1f. Mass spectrum of Impurity-3 at LOD level

Table 1. Chemical name, structure and molecular mass of Abacavir sulfate and its PGI's

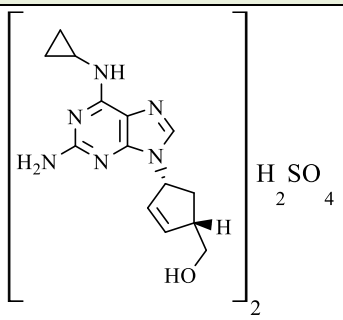
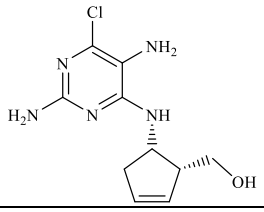
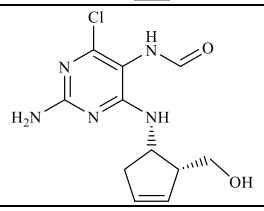
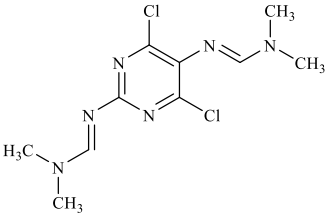
Compound	Chemical Name	Structure	Nominal Mass (In Da)
Abacavir Sulfate	{(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methanol		671
Impurity- I	{(1*S*,4*R*)-4-[(2,5-Diamino-6-chloropyrimidin-4-yl)amino]cyclopent-2-enyl}methanol		255
Impurity-II	*N*-(2-Amino-4-chloro-6-{[(1*S*,4*R*)-4-(hydroxymethyl)cyclopent-2-en-1-yl]amino}pyrimidin-5-yl)formamide		283
Impurity-III	*N'*,*N'*-(4,6-dichloropyrimidin-2,5-diyl)bis[*N*,*N*-imethyl(imidoformamide)]		288

Table 2. Analytical method validation data

Parameter	Result		
	Impurity-I	**Impurity-II**	**Impurity-III**
Detection Limit (ppm)	0.25	0.24	0.23
Quantitation Limit (ppm)	0.74	0.73	0.73
Linearity Range (ppm)	0.74-3.63	0.73-3.64	0.73-3.64
Slope	461398	318489	638883
Intercept	-25189	929	33546
Correlation Coefficient	0.999	0.999	0.999
Precision at LOQ Level (% RSD)	0.89	1.15	0.95
System Precision (% RSD)	1.76	1.04	2.73
Method Precision (% RSD)	1.25	0.85	1.05
% Recovery at LOQ Level	88.7-95.0	93.0-99.3	112.9-115.0
% Recovery at 100% Level	104.0-105.5	88.9-95.8	88.9-100.1
% Recovery at 150% Level	100.1-101.5	87.9-98.5	100.8-101.5

Table 3. Robustness

S. No.	Name of component					
		Actual condition (% RSD)	**Flow decrease (% RSD)**	**Flow increase (% RSD)**	**Temperature decrease (% RSD)**	**Temperature increase (% RSD)**
1	Impurity-I	1.02	1.10	0.92	0.90	0.85
2	Impurity-II	2.01	1.95	1.15	2.15	1.55
3	Impurity-III	1.05	1.15	1.20	0.98	1.65

S. No.	Name of component	Ionization energy		
		Actual condition 4.0Kv (% RSD)	**Temperature increase 4.5Kv (% RSD)**	**Temperature decrease 3.5Kv (% RSD)**
1	Impurity-I	0.95	0.84	1.02
2	Impurity-II	1.45	1.65	1.95
3	Impurity-III	1.85	1.52	1.46

Conclusion

A new validated method using LC-MS has been presented for the determination of three PGI's namely Impurity-I, Impurity-II and Impurity-III, at their limit of TTC. All the critical method parameters have been investigated and optimized. The method has been duly validated as per ICH guidelines. In light of the growing concern over the presence of PGI's in drug substance, this method can be used for routine analysis of ABA to determine the content of PGI's and ensure quality as well as regulatory compliance.

Acknowledgement

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