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Original Article

A Simultaneous Quantification of Four Potential Genotoxic Impurities Purinediol Hydrochloride, N7-Isomer, Monoalkyl Contaminant and Diacetyl Purine Present in Penciclovir Using LC-MS/MS

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ABSTRACT

Background: Penciclovir is a medication for antiviral therapy for curing different kinds of herpes virus infections. The four contaminants are the starting materials of Penciclovir which are present at a trace level in the drug in which one of its impurities is its isomer. Purinediol Hydrochloride, N7-Isomer, Monoalkyl contaminant and Diacetyl Purine are the four potential impurities present in the drug. **Materials and Methods:** The separation was achieved in the Zorbax SB C8 column using a gradient elution of 10 mM Ammonium bicarbonate in water as a buffer and a mixture of Methanol and Acetonitrile in the ratio of 20:80 v/v at a flow rate of 0.6 mL/min at 40ºC. The Agilent Q-TOF 6540 series instrument was used in positive polarity in the scan range of 50-100 *m/z*. The sensitive and rapid Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) technique was developed for the quantification of four impurities present in the drug. **Results:** Various validation parameters have been studied. The correlation coefficients of impurities and their linearity values are quite satisfactory within the examined range. The proposed method was sensitive in the linearity range from 0.6 ppm to 12.0 ppm. **Conclusion:** This method can be used to identify the impurities in Penciclovir drug substances during manufacturing.

Keywords: Penciclovir, Genotoxic impurities, LC-MS/MS method, Multiple Reaction Monitoring (MRM), Method Development and Validation.

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INTRODUCTION

Antiviral medications are usually employed to prevent or treat the herpes simplex virus. Penciclovir is such a drug that helps with antiviral treatment. It is an analogue of the nucleoside guanosine used to treat various kinds of herpes virus infections. It is distinguishable and less toxicity. Penciclovir has an interfering with DNA polymerase, thereby decreasing the virus's capability for growth. It effectively works against Types 1 and 2 of the herpes simplex virus, Epstein virus and hepatitis B virus, SARS COVID-2[1] as well as the varicella-zoster virus.[2]

Penciclovir is chemically known as 2-[2- (2-amino-6-hydroxy-9 H-purin-9-yl) ethyl] propane-1, 3-diol (or) 9- [4-hydroxy-3-(hydroxyl methyl) butyl] guanine. Its molecular formula is $C_{10}H_{15}N_5O_3$ with a molecular weight of 253.26.[3] It is comparatively less toxic and possesses better distinction.

When manufacturing pharmaceutical products to be used commercial purpose or for research purposes, it is the fundamental responsibility of druggists, chemical technology experts, inventors and formulators to certify that their production is safe.[4] So the raw materials used to manufacture pharmaceutical products must consider the high quality and high purity of the materials for safety and best results. The final pharmaceutical products in the synthesis process may contain impurities like starting substances, solvents, reagents, intermediate products or reaction by-products. These contaminants may have the adequacy to interface with DNA and RNA, the building blocks of hereditary or genetic material and induce gene mutations and chromosome breaks as well as chromosome rearrangements that develop cancer in humans[5-7] and these impurities were termed as Potential Genotoxic Impurities (PGIs).[8] The impurity can also be classified as a genotoxic impurity if it has a structural warning for mutagenicity.[9] Drug manufacturers are paying attention to the

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control and limit of Genotoxic Impurities (GTIs) in drugs that could be harmful to our DNA and human health.

Because of its importance, global regulatory bodies such as the European Medicines Agency released guidelines on the control of the amount of GTI, The United States Food and Drug Administration (USFDA) and the ICH International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [ICH M7] have also framed guidelines for genotoxic impurities in the drug substance. These rules and regulations suggest a value for a threshold limit of 1.5 g/day of toxicological concern for genotoxic contaminants in pharmaceutical products.[10-13] Therefore, it is not possible to remove genotoxic impurities completely from pharmaceutical drugs. So, it is essential to reduce the amount of these genotoxic impurities to the greatest extent possible in the drugs.[12]

The Purinediol, N7-Isomer of Penciclovir, Mono alkyl Contaminant and Diacetyl Purine are the key starting materials in the synthesis of Penciclovir. The compounds Purinediol, N7-Isomer of Penciclovir, Mono alkyl Contaminant and Diacetyl Purine are the gene toxic contaminants in the Penciclovir antiviral drug. The chemical structure, IUPAC name and molecular weight of Penciclovir and its Gene toxic contaminants are shown in Figure 1.

The thorough literature survey revealed that several articles describe the quantification of Penciclovir by LC, HPLC, RPLC and LCMS/MS.[15-23] However, no such literature has been published on a distinctive, rapid and susceptive LC-MS/MS methodology for the identification of four gene contaminants Purinediol, N7-Isomer of Penciclovir, Mono alkyl Contaminant and Diacetyl Purine in Penciclovir drug. Detection and quantification of such low-level impurities during the synthesis of pharmaceutical products are remarkably challenging. Thus, a new and valid method must be developed for the detection and quantification of low-level genotoxic impurities in pharmaceutical products. The primary objective of the current developed approach was the selective, sensitive, and accurate identification of four gene contaminants in Penciclovir drugs (Purine diol, N7-isomer of Penciclovir, mono alkyl contaminants and Diacetyl purine) using the LC-MS/MS approach. This approach was validated as per ICH guidelines in terms of the Limit of Detection (LOD), the Limit of Quantification (LOQ), linearity, precision, robustness and solution stability.[24]

MATERIALS AND METHODS

Fortune Pharma, Hyderabad, provided gift samples of Penciclovir and its starting materials as API (99.1%). HPLC grade methanol and Acetonitril were purchased from Merck, Mumbai, India. HPLC grade water was prepared by a millipore purification system. AR grade Ammonium bicarbonate was procured from SD Fine chemicals, Mumbai, India.

Optimized chromatographic conditions of LC-MS/MS method

The procedure was carried out with an Agilent Q-TOF 6540 series instrument operated for MS/MS parameters. Zorbax SB C8 column (100 mmX4.6 mm, 3.5 μm) using gradient elution was used for separation with 10 Mm Ammonium bicarbonate in water as buffer and a mixture of Methanol and Acetonitrile in the ratio 20:80 v/v at a flow rate of 0.6 mL/min and the column temperature was maintained at 40ºC. The Zorbax SB C8 column was selected because of its stable reversed-phase packing, which can accommodate samples of basic, neutral, acidic and neutral at low and high pH levels. All mass parameters were incorporated in Table 1. The Figure 2 displays the mass spectra's of impurities Purine diol hydrochloride, N7-Isomer, Mono alkyl Contaminant and Diacetyl purine.

Instrumentation and Mass Spectrometer Conditions in LC- MS/MS (Optimized Methodology) Chromatographic Parameters

The desolvation temperature and source temperature were maintained at 500ºC and 150ºC respectively. MRM (Multiple Reaction Monitoring) modes were selected for the determination of Purinediol, N7-Isomer of Penciclovir, Mono alkyl Contaminant and Diacetyl purine, the transitions at 308.16 *m/z*, 253.26 *m/z*, 223.24 *m/z* and 355.78 *m/z* (parent mass) and 185.92 *m/z*, 176.86 *m/z*, 154.35 *m/z* and 197.91 *m/z* (fragment mass) were selected on the basis of response.

Preparation of Diluents

Prepare a filtered and degassed the mixture of Methanol: buffer (80:20) v/v.

Preparation of Ammonium Bicarbonate Buffer

Prepare 0.79 g of Ammonium bicarbonate in 1000 mL of water and filtered through a 0.22 μm or finer porosity membrane and degassed.

Preparation of Standard Solutions

Accurately weighed and transferred the appropriate amount of contaminants (Purinediol hydrochloride, N7-Isomer, Mono alkyl Contaminant, Diacetyl Purine) to get a concentration of 4.0 μg/mL solution, which is equivalent to 8.0 ppm with respect to the drug. The linearity solutions were prepared at 0.6, 1.64, 1.67, 2.13, 4.0, 6.0, 8.0, 10.0 and 12.0 ppm level concentrations with respect to the drug.

Preparation of Spiked Sample Solution

Prepare the spiked sample solution containing 0.5 mg/mL of Penciclovir in a volumetric flask, dissolve it, and dilute it with the standard solution.

Methodology Verification

The methodology precision was evaluated by preparing six spiked sample solutions at the specified limit of contaminants and determined the % RSD. By analyzing the six-point calibrators, the linearity can be evaluated. By the process of analysing the spiked sample solution at various instances at normal temperatures, we can analyse the stability of those contaminants.

Name of the compound: Penciclovir Molecular Formula: $C_{10}H_{15}N_5O_3$ Molecular Weight: 253.26 IUPAC Name: 2-[2-(2-amino-6-hydroxy-9H-purin-9-yl)ethyl] propane-1,3-diol (or) 9-[4-hydroxy-3-(hydroxyl methyl) butyl] guanine	
Name of the compound: Purine diol hydrochloride Molecular Formula: $C_{10}H_{15}Cl_2N_5O_2$ Molecular Weight:308.16 IUPAC Name: 2-[2-(2-amino-6-chloro-9H-purin-9-yl) ethyl] propane-1,3-diol hydrochloride	Name of the compound: N7-Isomer Molecular Formula: $C_{10}H_{15}N_5O_3$; Molecular Weight: 253.26 IUPAC Name: 2-[(acetyl oxy) methyl]-4-(2-amino-6-chloro-7H-purin-7-yl)butyl acetate)
Name of the compound: Mono alkyl Contaminant Molecular Formula: $C_9H_{13}N_5O_2$; Molecular Weight: 223.24 IUPAC Name: 2- amino-9-(4-hydroxy butyl) -9H-purin-6-ol.	Name of the compound: Diacetyl purine Molecular Formula: $C_{11}H_{18}ClN_5O_4$; Molecular Weight: 355.78 IUPAC Name: 2-[(acetyl oxy) methyl]-4-(2-amino-6-chloro-9H-purin-9-yl) butyl acetate.

Figure 1: Chemical Structure of Penciclovir and its Genotoxic Contaminants.

RESULTS

Developing Analytical Methodology for Gene toxic Contaminants

This optimised methodology fulfilled the criteria of system suitability. Administer diluents as blank followed by standard solution, six times into the chromatograph and record the chromatograms. The system is suitable if and only if, The Signal to Noise ratio of contaminants should not be less than 30 from the first administration of the standard solution. The percentage relative standard deviation for the peak area of contaminants should not be more than 15% from six replicate administrations.

Methodology Verification study for Gene toxic Contaminants Particularity

Penciclovir was prepared along with a standard concentration of each individual contaminant. Additionally, a solution of Penciclovir spiked with contaminants was prepared and added to the chromatographic system. The particularity of the data Retention time (min) and observed mass ((M+H)+) values for Purinediol hydrochloride are 5.19 and 308.16, respectively. Similarly, the values for N7-Isomer are 4.26 and 253.26, for Monoalkyl Contaminant are 6.24 and 223.24, for Diacetyl Purine are 7.69 and 355.78 and for Penciclovir are 3.61 and 254. The typical chromatogram blank, standard, sample, spiked sample and mass spectra were represented in Figure 3a, 3b and 3c.

DISCUSSION

Linearity

The linearity study of gene toxic contaminants was performed with a seven-point linear calibration graph between 0.6 ppm and 12.0 ppm of contaminants. The linearity of peak area versus concentration was demonstrated to be LOQ-150% level of contaminants. The information was subjected to statistical investigation using linear regression at least square methodology. The calibration curve was found to be linear with the correlation

Table 1: Mass Parameters of Genotoxic contaminants.

Name of the Impurity	Mode	Parent Ion (*m/z*)	Daughter Ion (*m/z*)	Cone (Volts)	Collision (Volts)
Purine diol hydrochloride impurity	+ve	308.16	185.92	8	21
N7-Isomer impurity	+ve	253.26	176.86	12	30
Mono alkyl Contaminant impurity	+ve	223.24	154.35	60	14
Diacetyl purine impurity	+ve	355.78	197.91	24	10

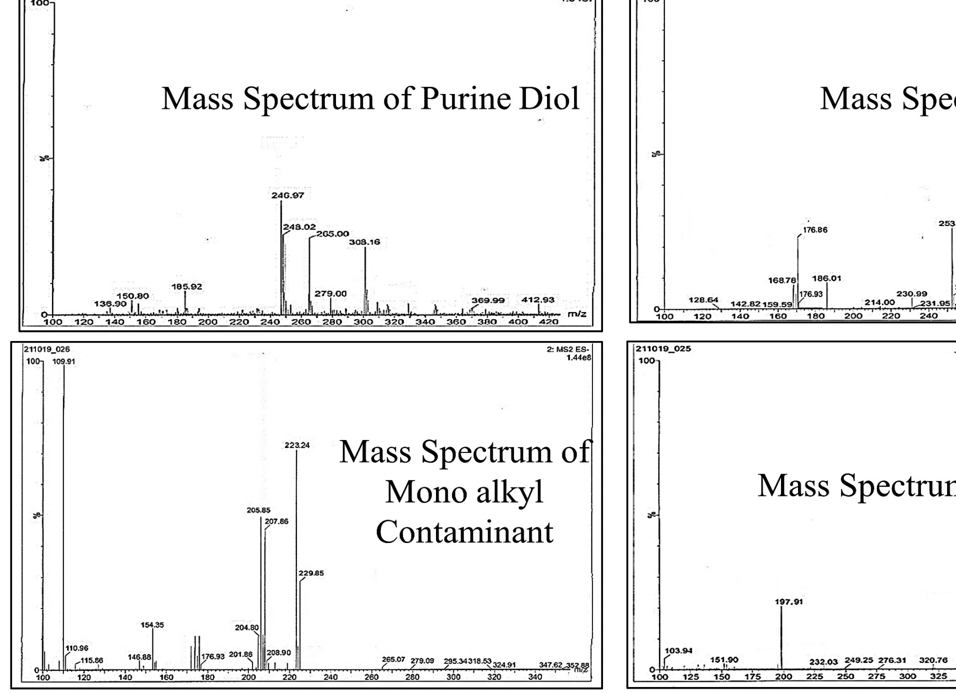

Figure 2: 2a. Mass spectrum of Purine Diol, 2b. Mass spectrum of N7 Isomer, 2c. Mass spectrum of Mono alkyl contaminant and 2d. Mass spectrum of Diacetyl purine.

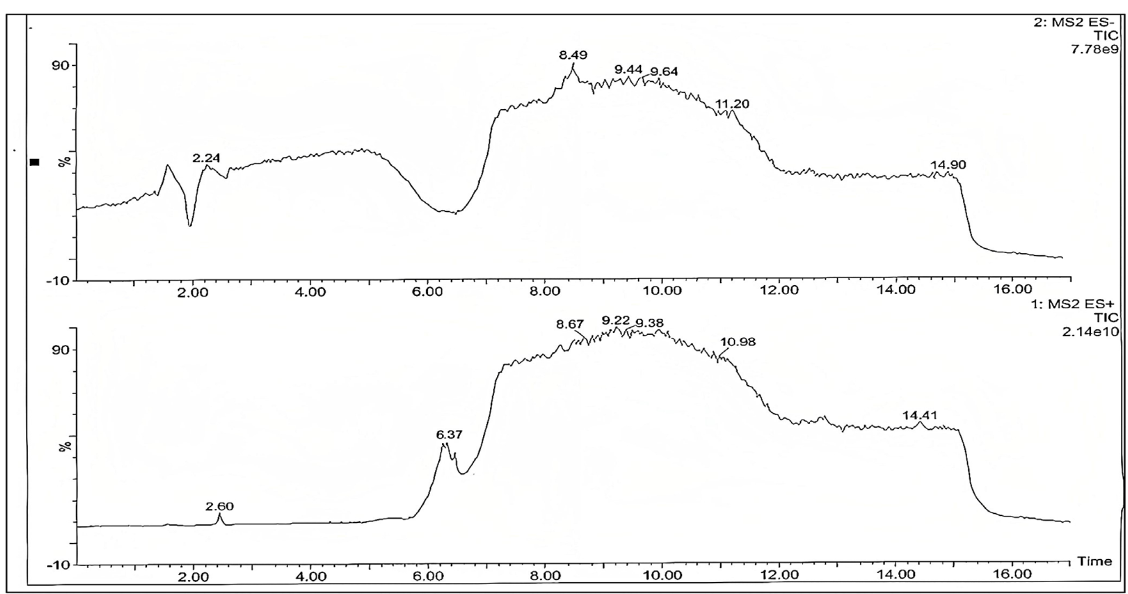

Figure 3a: Typical Mass Spectrum of Blank.

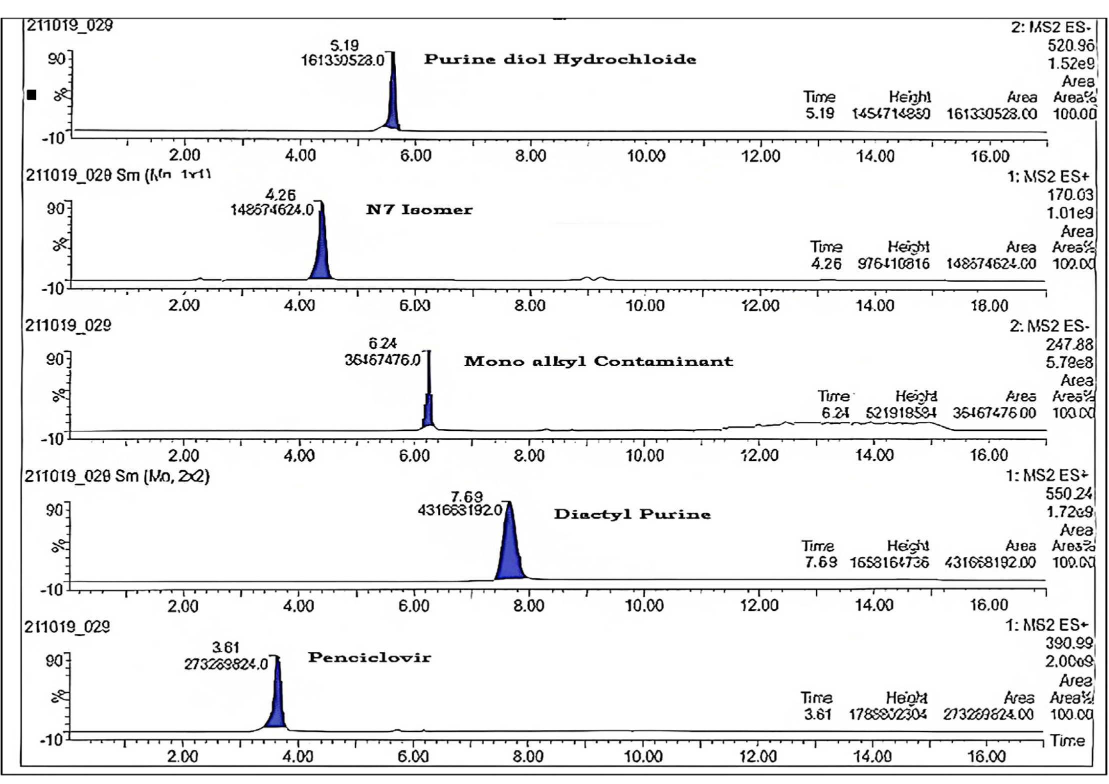

Figure 3b: Typical particularity Spectrum of Penciclovir and its Contaminants.

effective (R) shown in Figure 4 and the results are given in Table 2.

Limit of Quantification and Limit of Detection

The Signal-to-Noise ratio (S/N) approach was used to determine the LOD (Limit of Detection) and LOQ (limit of quantification) for the impurities. The standard solution for the impurities was further diluted to achieve an S/N ratio of nearly 10:1 for the LOQ. The LOQ solution was further diluted three times to achieve an S/N ratio of nearly 3:1 for the LOD. The Limit of detection of Purine diol hydrochloride, N7-Isomer, Mono alkyl Contaminant and Diacetyl Purine were found to be 0.55 ppm, 0.20 ppm, 0.70

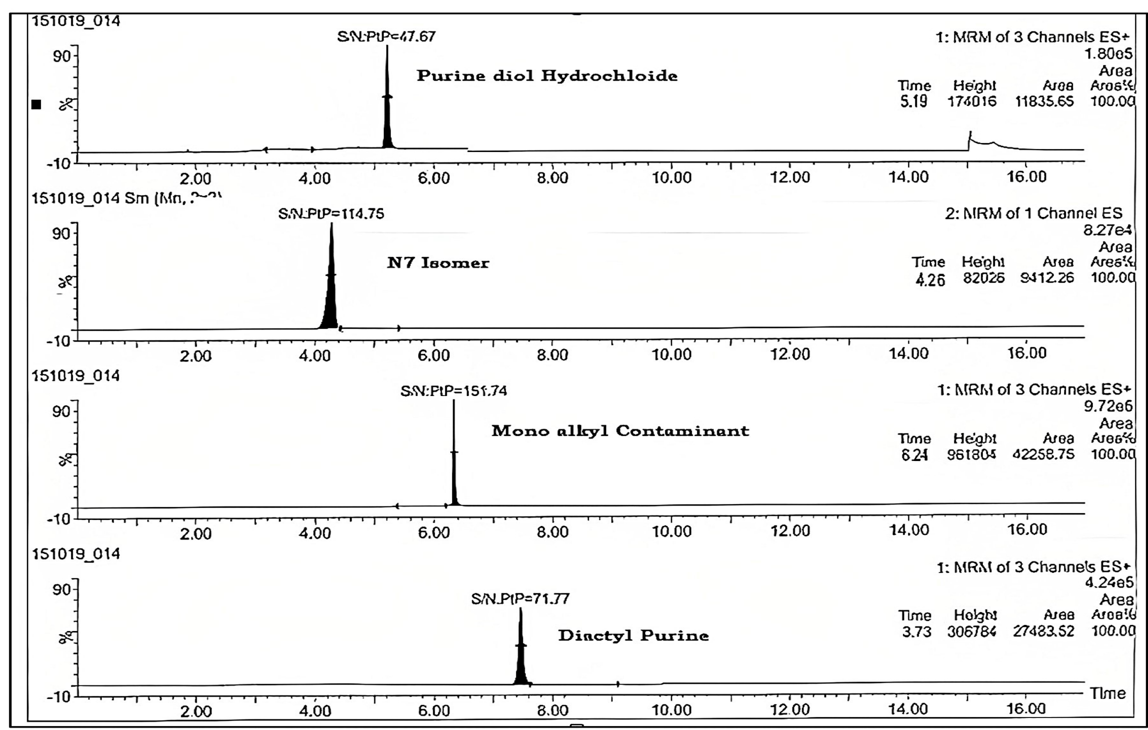

Figure 3c: MRM spectrum of Penciclovir Gene toxic Contaminants.

Figure 3: 3a.Typical mass spectrum of blank 3b.Typical particularity spectrum, 3c.MRM spectrum.

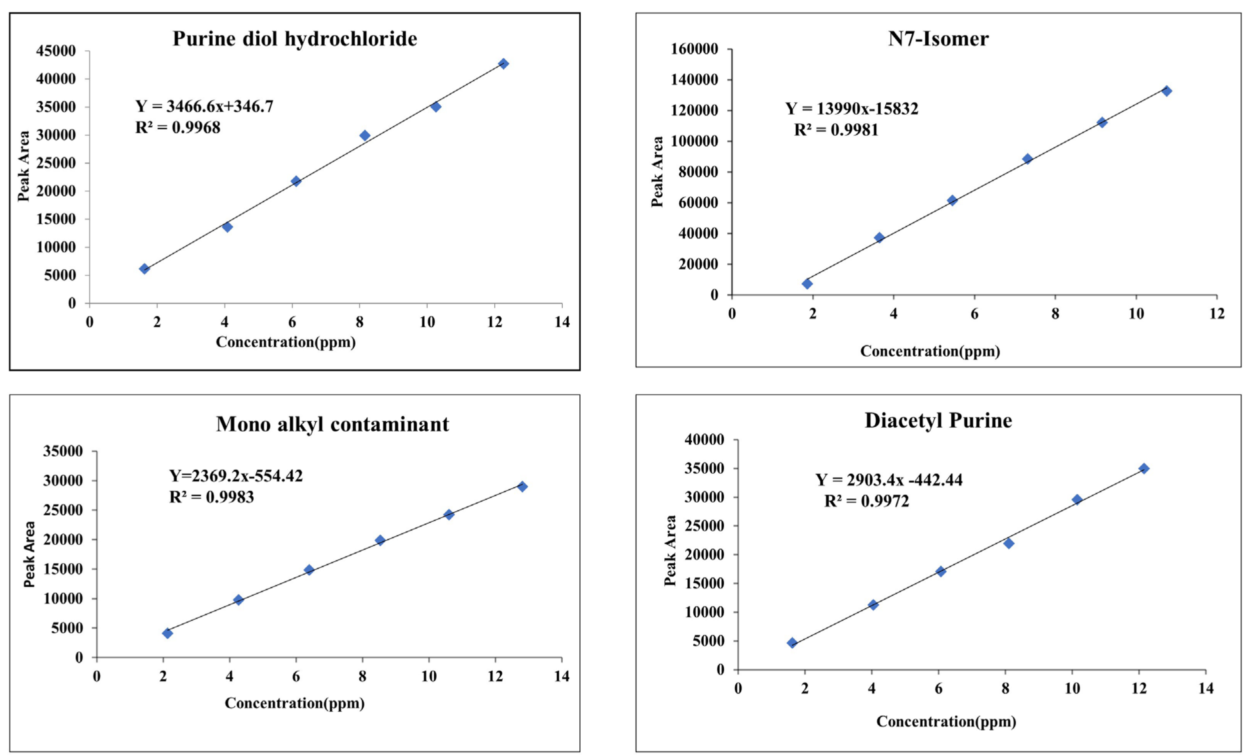

Figure 4: Linearity curves of Penciclovir and its Genotoxic contaminants.

Table 2: Linearity of Penciclovir Gene toxic Contaminants.

2a. Penciclovir Gene toxic Contaminants				
Component	**Slope**	**Intercept**	**Correlation Coefficient (R)**	$_{R}2$
Purine diol hydrochloride	3466.6	346.7	0.9968	0.9968
N7-Isomer	13990	15832	0.9981	0.9981
Mono alkyl Contaminant	2369.2	554.42	0.9983	0.9983
Diacetyl purine	2903.4	442.44	0.9972	0.9972

2b. Purine diol hydrochloride			
% Level	**Against particulation**	**Concentration (ppm)**	**Purinediol hydrochloride**
LOQ	19.8	1.624654E+00	6154
50	49.5	8.154564E+00	29890
75	73.9	4.084654E+00	13589
100	98.2	6.121654E+00	21786
125	123.9	1.025646E+01	35052
150	148.3	1.226046E+01	42695

2c. N7-Isomer			
% Level	**Against particulation**	**Concentration (ppm)**	**N7-Isomer**
LOQ	7.5	1.860312E+00	7269
50	43.8	7.311664E+00	88525
75	66.2	3.650215E+00	37204
100	89.5	5.4501651E+00	61358
125	109.9	9.154012E+00	112057

2d. Mono alkyl Contaminant			
% Level	**Against particulation**	**Concentration (ppm)**	**Monoalkyl contaminant**
LOQ	24.8	2.130231E+00	4125
50	50.4	8.541655E+00	19862
75	76.7	4.271231E+00	9803
100	101.5	6.403351E+00	14896
125	127.9	1.060321E+01	24215

2e. Diacetyl Purine			
% Level	**Against particulation**	**Concentration (ppm)**	**Diacetyl Purine**
LOQ	19.5	1.619051E+00	4652
50	48.8	8.095455E+00	21987
75	73.2	4.045412E+00	11254
100	97.5	6.071515E+00	17056
125	121.9	1.014598E+01	29561
150	146.3	1.214652E+01	34985

Table 3: Accuracy results of four contaminants.

3a. Purine diol hydrochloride			
% Level	% Recovery		
	Individual	Average	% RSD
LOQ	99.5	98.93	0.40
	98.6		
	98.7		
50	101.2	101.60	0.62
	102.5		
	101.1		
150	102.4	103.16	1.93
	105.9		
	101.2		
150	100.5	101.43	0.69
	101.6		
	102.2		
3b. N7-Isomer			
% Level	% Recovery		
	Individual	Average	% RSD
LOQ	104.1	105.53	1.02
	105.8		
	106.7		
50	107.5	105.1	1.70
	103.2		
	104.6		
150	103.1	103.63	0.48
	104.3		
	103.5		
150	107.5	106.56	0.81
	106.8		
	105.4		
3c. Mono alkyl Contaminant			
% Level	% Recovery		
	Individual	Average	% RSD
LOQ	103.2	103.53	0.51
	107.8		
	103.3		
50	104.2	103.42	2.47
	99.98		
	106.1		
150	100.2	100.65	0.44
	101.1		
	105..3		

150	102.4	103.1	1.17
	102.1		
	104.8		
3d. Diacetyl purine			
% Level	**% Recovery**		
	Individual	**Average**	**% RSD**
LOQ	104.2	103.53	0.51
	103.5		
	102.9		
50	101.2	103.3	2.94
	107.6		
	101.1		
150	106.2	103.86	1.93
	104.1		
	101.3		
150	104.2	102.7	1.06
	101.6		
	102.3		

Table 4: Precision results of four contaminants.

System Precision				
Administration No.	**Purinediol hydrochloride**	**N7-Isomer**	**Mono alkyl Contaminant**	**Diacetyl purine**
Administration 1	26859	42569	8169	11065
Administration 2	25798	42981	8681	11656
Administration 3	25685	43265	8625	11326
Administration 4	26562	42054	8661	11274
Administration 5	25168	42167	8667	10987
Administration 6	26135	42255	8653	11765
Average	26034.5	42548.5	8576	11345.5
% RSD	2.15	1.04	2.13	2.50
Methodology Precision				
Preparation	**Purine diol hydrochloride**	**N7-Isomer**	**Mono alkyl Contaminant**	**Diacetyl Purine**
Preparation 1	8.09	8.21	8.16	8.29
Preparation 2	8.31	8.16	8.31	8.65
Preparation 3	8.27	8.42	8.54	8.43
Preparation 4	8.13	8.31	8.18	8.36
Preparation 5	8.23	8.24	8.29	8.85
Preparation 6	8.37	8.23	8.13	8.19
Average	8.23	8.26	8.263	8.46
% RSD	1.18	1.01	1.67	2.64

ppm, and 0.54 ppm respectively. The limit of quantification of Purine diol hydrochloride, N7-Isomer, Monoalkyl Contaminant and Di acetyl purine were found to be 01.67 ppm, 0.60 ppm, 2.13 ppm and 1.64 ppm respectively.

Accuracy

A recovery study of penciclovir spiked with known contaminants was carried out in triplicate at LOQ 50%, 100% and 150% levels of standard solution concentration. The percentage recovery of contaminants has been calculated and found to be within the range of 80-120%. The results of the accuracy of gene toxic contaminants were represented in Table 3.

Precision

By analysing the six administrations of standard solutions, we determine the system precision. The results of percent standard deviations are in the range of 0.3-2.3%. The methodology precision was performed by analysing six sample preparations

Table 5: Robustness Study with Respect to Flow and Column oven Temperature.

Parameter Condition	Purinediol hydrochloride	N7-Isomer	Mono alkyl Contaminant	Diacetyl purine
Actual (Flow: 0.6 mL/min; Temperature 40ºC)	8.07	8.21	8.09	8.21
Flow rate: 0.55 mL/min (Low Flow)	8.25	8.15	8.52	8.65
Flow rate: 0.65 mL/min (High Flow)	8.35	8.18	8.41	8.55
Column oven temperature 38ºC (Low)	8.24	8.18	8.09	8.48
Column oven temperature 42ºC (High)	8.21	8.35	8.21	8.09

Table 6: Solution stability Study at Normal Temperature (24ºC to 26ºC and 2-8ºC).

Component	Temperature Conditions	Solutions	Initial (ppm)	After 24 hr (ppm)	After 48 hr (ppm)
Purine diol hydrochloride	Normal temperature (24-26ºC)	Standard	8.04	8.84	9.01
		Sample	Not detected	Not detected	Not detected
		Spiked	8.09	8.35	8.56
	2-8ºC	Standard	8.05	8.92	9.08
		Sample	Not detected	Not detected	Not detected
		Spiked	8.10	8.52	8.46
N7-Isomer	Normal temperature (24-26ºC)	Standard	8.05	8.79	8.62
		Sample	Not detected	Not detected	Not detected
		Spiked	8.09	8.65	8.51
	2-8ºC	Standard	8.05	8.58	8.60
		Sample	Not detected	Not detected	Not detected
		Spiked	8.10	8.69	8.72
Mono alkyl Contaminant	Normal temperature (24-26ºC)	Standard	8.05	8.51	8.56
		Sample	Not detected	Not detected	Not detected
		Spiked	8.12	8.58	8.65
	2-8ºC	Standard	8.05	8.51	8.91
		Sample	Not detected	Not detected	Not detected
		Spiked	8.13	8.66	9.03
Diacetyl purine	Normal temperature (24-26ºC)	Standard	8.07	8.96	8.64
		Sample	Not detected	Not detected	Not detected
		Spiked	8.10	8.88	8.49
	2-8ºC	Standard	8.05	8.94	8.87
		Sample	Not detected	Not detected	Not detected
		Spiked	8.12	8.62	8.71

of penciclovir spiked with gene toxic contaminants at standard concentrations. The % relative standard deviation values of methodology precision were found in the range of 1.1-4.1%. The results of system precision and methodology precision are represented in Table 4.

Robustness

The robustness of the developed methodology was performed for the contaminants by introducing minuscule changes in the chromatographic conditions, which includes mobile phase variation (altered by10% of flow) and column temperature (altered by ±2ºC temperature) and the impact of the change was observed on the chromatographic performance. The methodology has proven robust by making minor changes to the chromatographic parameters. The results of the robustness study of retention time and % w/w of contaminants were summarised in Table 5.

Solution Stability

A penciclovir solution spiked with the gene toxic contaminants at ICH particulation level concentration and standard solutions were kept at 24-26ºC (normal temperature) and at 2-8ºC (refrigerator). The stability of the solution was verified at 0 hr (initial), after 24 hr and at 48 hr intervals and comparing the results indicates that the contaminant's standard solution and sample solutions were stable up to 48 hr at 2-8ºC as well as at normal temperature (25ºC). The solution stability results of Penciclovir and its contaminants were summarised in Table 6.

CONCLUSION

The gene toxic contaminants (Purine diol hydrochloride, N7- Isomer, Monoalkyl Contaminant and Diacetyl Purine) in penciclovir drug have been quantified employing the LC-MS/MS technique in this study. It was a susceptive, rapid, distinctive, accurate, linear, precise, robust and cost-effective methodology. The LC-MS/MS methodology is well confined to the limits of ICH guidelines for the investigation of genotoxic contaminants in Penciclovir. The limit of detection was found to be as low as 0.55 ppm, 0.20 ppm, 0.70 ppm, and 0.54 ppm indicating high susceptivity. The proposed methodology was used for the investigation of gene toxic contaminants in drugs.

ACKNOWLEDGEMENT

The authors are thankful to Fortune Pharma Lab, Hyderabad for supporting and providing the analytical instruments, chemicals and solvents for the simultaneous Quantification of four potential Genotoxic Impurities Purine diol Hydrochloride, N7-Isomer, Monoalkyl contaminant and Diacetyl Purine present in Penciclovir using Liquid Chromatography coupled with Tandem Mass Spectrometer.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MRM: Multiple Reaction Monitoring; **ICH:** International Council for Harmonisation; **USFDA:** United States Food and Drug Administration; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **PPM:** Parts Per Million; **RSD:** Relative Standard Deviation.

SUMMARY

The current study explains how to quantify penciclovir and its genotoxic contaminants purine diol hydrochloride, N7-isomer, Monoalkyl pollutants and Diacetyl purine by the LC-MS/MS technique. The identification of genotoxic contaminants in the LC-MS/MS technique was based on their mass in the LC-MS/MS chromatogram. The method was developed on a Zorbax SB C8 column (100 mmX4.6 mm, 3.5 µm) using gradient elution for separation with 10 mM ammonium bicarbonate in water as a buffer and a mixture of Methanol and Acetonitrile in the ratio 20:80 v/v at a flow rate of 0.6 mL/min, and the column temperature was maintained at 40ºC. The developed method was validated under ICH Q2 (R2) standards. The developed method is easy, innovative, accurate, specific, effective, robust and inexpensive. Regular analysis of this drug can be done in quality control and research labs using this technique.

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