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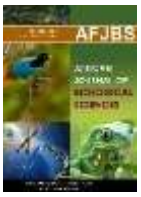

ADVANCED ANALYTICAL METHODS: LC-MS/MS WITH MULTIPLE REACTION MONITORING (MRM MODE) FOR THE QUANTIFICATION OF TWO POTENTIAL GENOTOXIC IMPURITIES IN EMTRICITABINE DRUG SUBSTANCE

Pantula Nagendra Srinivas [1*], Shyamala Pulipaka[2] , Nagaraju Cherukuru[1]

[1]Aurobindo Pharma Limited, Department of Chemistry, Andhra Pradesh, Visakhapatnam

[2] Department of Chemistry, Andhra University, Visakhapatnam Correspondng author e-mail: pnsrinivas70@gmail.com

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Abstract:

Sensitive LC-MS/MS techniques were developed and verified for 2-Methoxy 4-Amino-5-Fluro pyramidine and L-menthyl glyoxalate contaminants in the active pharmaceutical ingredient (API) of Emtricitabine.Ultra Performance Liquid Chromatography, Acquity H-Class system, with a Quaternary Solvent manager, gradient mixer assembly, Sample Manager - F.T.N (Flow through needle) auto injector, with a column oven coupled to Xevo TQS Triple Quadrupole LC/MS/MS Mass Spectrometer, (Make Waters).Column was employed in the method was Acquity UPLC CSH Phenyl Hexyl 1.7 µm. (150mm x 2.1mm) and X-Select CSH C18 150x4.6 3.5µm mm respectively. All the weighing in the experiments was done with Sartorius balance capable of measuring with an accuracy of 0.01 mg.The method was developed and validated as per of ICH guideline. The suggested approach was robust, accurate, linear, and specific. Over the concentration range of 0.6 µg/g to 9.5 µg/g and 0.6 µg/ml to 9.4 µg/ml, the calibration curves demonstrated satisfactory linearity; the correlation coefficients were.0.999 and.0.999 in each case. The method's very low limit of quantification (LOQ) and limit of detection (LOD) were 0.3 µg/g and 0.6 µg/g, respectively.Analytical test method for determination of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine in Emtricitabine by LC-MS/MS was validated for System Suitability, Identification, Specificity, Linearity, Method Precision,

Keywords: Genotoxic impurities, LC-MS/MS , MRM , Emtricitabine ,Validation, ICH.

Introduction:

A nucleoside reverse transcriptase inhibitor called emtricitabine is used to treat HIV (human immunodeficiency virus) infection in adults[1-4] The medication copies HIV RNA (ribonucleic acid) into new virus DNA (deoxyribonucleic acid) by suppressing the reverse transcriptase enzyme. Tenofovir disoproxil fumarate is frequently given in conjunction with emtricitabine; the daily maximum dose of emtricitabine is 0.2 g. 2-Methoxy 4-Amino-5-Fluro pyramidine and L-menthyl glyoxalate contaminants in the active pharmaceutical ingredient (API) of Emtricitabine.

Based on the maximum daily dosage of Emtricitabine L -Menthyl glyoxylate monohydrate (LMGH) and 2-Methoxy 4-Amino-5-Fluro pyramidine (MAFP) are required to be controlled at a limit of NMT 6.25 µg/g and NMT 6.25 µg/g respectively in the API.

For the determination of L -Menthyl glyoxylate monohydrate (LMGH) and 2-Methoxy 4-Amino-5-Fluro pyramidine (MAFP), the suggested LC-MS/MS (liquid chromatography/mass spectrometery/mass spectrometery) approach provides a straightforward, robust, and labor-saving alternative that requires no time-consuming sample preparation steps. The suggested technique uses electrospray ionization in the MRM mode to quantify L -Menthyl glyoxylate monohydrate (LMGH) and 2-Methoxy 4-Amino-5-Fluro pyramidine (MAFP).Emtricitabine and its impurities structures shown in (figure-1).

Material and Methods:

Chemical & Reagents:

Instrumentation:

An Ultra Performance Liquid Chromatography, Acquity H-Class system, with a Quaternary Solvent manager, gradient mixer assembly, Sample Manager - F.T.N (Flow through needle) auto injector, with a column oven coupled to Xevo TQS Triple Quadrupole LC/MS/MS Mass Spectrometer, (Make Waters).Column was employed in the method was Acquity UPLC CSH Phenyl Hexyl 1.7 µm. (150mm x 2.1mm) and X-Select CSH C18 150x4.6 3.5µm mm respectively. All the weighing in the experiments was done with Sartorius balance capable of measuring with an accuracy of 0.01 mg. Chromatographic Conditions shown in Table No:1 List of Chemicals, Reagents, Chemicals, Sample, Standards and Impurities shown in Table No:2

Mass spectroscopic conditions:

Source Type : ESI
Mode of Ionization : Positive

SOURCE PARAMETERS:

Capillary : 3.9 kv
Source Temperature : 150° C
Desolvation temperature: 500° C
Cone gas flow : 150 L/Hr
Desolvation gas flow : 1000 L/Hr
Nebulizer (Bar) : 7.0
Condition for MRM:
Scan Type :MRM

Function type : MRM of 1 channel

Name	Q1Mass (amu)	Q3Mass (amu)	Cone(v)	Collision energy (v)
L-Methyl Glyoxalate monohydrate	253.2 $[M+Na]^+$	115.0	26	10
2-Methoxy 4-Amino-5-Fluro pyramidine	143.9	112.8	48	18

Preparation of Diluent for L-Methyl Glyoxalate monohydrate :

Prepare a degassed mixture of Methanol, Water and methanolic sodium hydroxide solution in the ratio of 800:200:0.1 v/v/v.

Preparation of Diluent for 2-Methoxy 4-Amino-5-Fluro pyramidine:

Prepare a degassed mixture of Acetonitrile, Water and Formic acid in the ratio of 500:500:0.1 v/v/v.

Preparation of Standard Solution for L-Methyl Glyoxalate monohydrate:

Primary standard stock solution:

Accurately weigh and transfer about 11.0 mg of L-Menthyl Glyoxalate reference sample into a 50 mL clean, dry volumetric flask, add 30 mL of diluent sonicate to dissolve and make up to volume with diluents (0.22mg/mL).

Pipette 3.0 mL of Primary standard stock solution into 50 mL of volumetric flask make up to the mark with diluents (528.0 µg/g). Further pipette 3.0 mL of solution into 50 mL of volumetric flask make up to the mark with diluents (31.68 µg/g). Further pipette 2.0 mL of solution into 10 mL of volumetric flask make up to the mark with diluent (6.34 µg/g). Specimen Chromatogram for Blank and L-Menthyl glyoxalate shown in (figure-2).

Preparation of Standard Solution for 2-Methoxy 4-Amino-5-Fluro pyramidine:

Primary standard stock solution:

Accurately weigh and transfer about 9.8 mg of 2-Methoxy 4-Amino-5-Fluro pyramidine reference sample into a 50 mL clean, dry volumetric flask, add 30 mL of diluent sonicate to dissolve and make up to volume with diluents (0.196 mg/mL).

Pipette 2.0 mL of Primary standard stock solution into 50 mL of volumetric flask make up to the mark with diluents (784.0 µg/g). Further pipette 2.0 mL of solution into 50 mL of volumetric flask make up to the mark with diluents (31.36 µg/g). Further pipette 2.0 mL of solution into 10 mL of volumetric flask make up to the mark with diluent (6.27 µg/g). Specimen Chromatogram for Blank and 2-Methoxy -4-amino-5-Fluoro pyrimidine shown in (figure-3).

Preparation of Sample solution for L-Methyl Glyoxalate monohydrate:

Accurately weigh and transfer 250 mg of sample into a clean, dry 10 mL volumetric flask/Centrifuge tube, add 5.0 mL of diluent sonicate to dissolve and then add 5.0 mL of diluent and mix well. Total volume is 10.0 mL

Preparation of Sample solution for 2-Methoxy 4-Amino-5-Fluro pyramidine:

Accurately weigh and transfer 100 mg of sample into a clean, dry 10 mL volumetric flask/Centrifuge tube, add 5.0 mL of diluent sonicate to dissolve and then add 5.0 mL of diluent and mix well. Total volume is 10.0 mL

Results & Discussion:

Validation:

System Precision:

Prepared the standard solution of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino 5-Fluoropyrimidine as per methodology.

%RSD for six standard solution injections were observed below 10.0% for both impurities and results are 1.8% for L-Menthyl Glyoxalate and 3.2% for 2-Methoxy 4-Amino 5-Fluoropyrimidine. System precision met the acceptance criteria. System precision results are shown in Table No:3

Identification and Specificity:

The identification and specificity is defined as the ability to assess and ensure that the impurities and diluent do not affect the sample analyzed. **Results for Identification shown in** Table No:4a and Table No:4b

For L-Methyl Glyoxalate monohydrate

Inject the Blank (as Diluent), Standard solution, and Control sample, Spiked sample with all related compounds with and without LMGM and at specification level. Check the interference at the retention time and mass of analyte.

For 2-Methoxy 4-Amino-5-Fluro pyramidine

Inject the Blank (as Diluent), Standard solution, and Control sample, spiked sample with all related compounds with and without MAFP and at specification level. Check the interference at the retention time and mass of analyte.

Spiked sample **r**etention times (RT) were comparable to that of reference sample for both impurities and both methods. .Blank and L-Menthyl glyoxalate LCMS Chromatograms. Hence methods can be capable for the identification and proved the specificity.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

Prediction of LOD & LOQ:

Prepared a series of diluted solutions of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities with respect to analyte concentration were prepared and injected into the LC-MS/MS system until to get the signal to noise (USP S/N) ratio is more than 3 for LOD and more than 10 for LOQ and achieved the target. The Signal to Noise (S/N) ratio of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine peaks were recorded using system data software. The LOQ and LOD results were captured in the below Table No:5

Precision at LOD & LOQ and Accuracy at LOQ:

Six different solutions were prepared to contain L-Menthyl Glyoxalate impurity and 2-Methoxy 4-Amino-5-Fluro pyramidine impurity separately as per methodology at proposed LOD & LOQ level and each solution was injected once into the L-MS/MS , area of L-Menthyl Glyoxalate impurity and 2-Methoxy 4-Amino-5-Fluro pyramidine impurity in each solution was recorded. %RSD for the content of L-Menthyl Glyoxalate impurity and 2-Methoxy 4-Amino-5-Fluro pyramidine impurity in each preparation was calculated. The % recovery of each solution was calculated and shown in Table No:6

S/N ratio, %RSD and Accuracy results were met the acceptance criteria.

Linearity:

Seven linearity solutions were prepared for L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-

Fluro pyramidine impurities from LOQ to 150% with respect to test concentration.

The Correlation Coefficient is more than 0.99 for L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities in both methodologies. Hence the response of for L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities is linear LOQ to 150% of specification level in both methods. Linearity plot of L-Menthyl Glyoxalate shown in (figure-4) and Linearity plot of 2-Methoxy 4-Amino-5-Fluro pyramidine shown in (figure-5) and shown Table No 7a and Table No 7b

Method Precision:

Six sample solutions were prepared individually by spiking the impurity into the Emtricitabine as per test method at specification level and injected into LC-MS/MS as per methodologies.

%RSD of area and obtained concentration for method precision met the acceptance criteria as per set criteria is not more than 20.0% for both L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities shown in Table No 8

Accuracy:

Prepared the samples spiked with L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities at 100% level and 150% level in presence of Emtricitabine drug substance (prepared in triplicates) against L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities at 100% level and 150% level in absence of Emtricitabine drug substance and injected into LC-MS/MS as per methodologies. Results for Accuracy LOQ Level for L-Menthyl Glyoxalate and Results for Accuracy LOQ Level for2-Methoxy 4-Amino-5-Fluro pyramidine shown in Table No:6a ,7a ,9,10a and 10b

The individual percentage recovery for each sample at 100% level & 150% specification level are meeting set criteria of between 80.0% to 120.0%.

Range:

Range of analytical method can be obtained from Linearity and Recovery data of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities in both methodologies. Reported the range in LOQ to 150% with respect to specification level.

Robustness:

Prepared standard solution as per test methods for L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine impurities at specification level. Injected to in to LC-MS/MS at deliberately varied conditions to evaluate the system suitability and methods ability to remain unaffected. The system suitability results at each of the varied conditions complied the requirements as per the test procedure. Hence it can be concluded that the test method is robust across the extent of changes studied for each of the above parameters.

Figure-1: structure of Emtricitabine it's related impurities

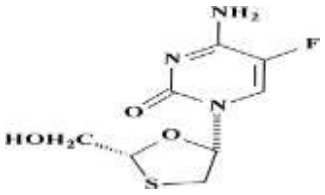
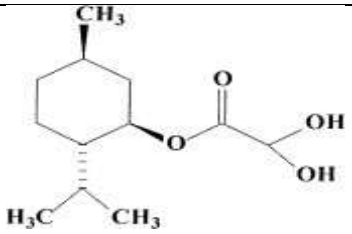
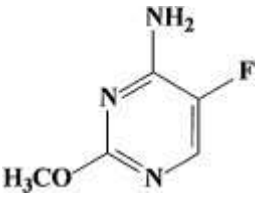
Compound Name	Structure
Emtricitabine	 Fig:1a
L-Menthyl glyoxylate monohydrate	 Fig:1b
2-Methoxy 4-Amino-5-Fluro pyramidine	 Fig:1c

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Figure 2: Specimen Chromatogram for Blank and L-Menthyl glyoxalate

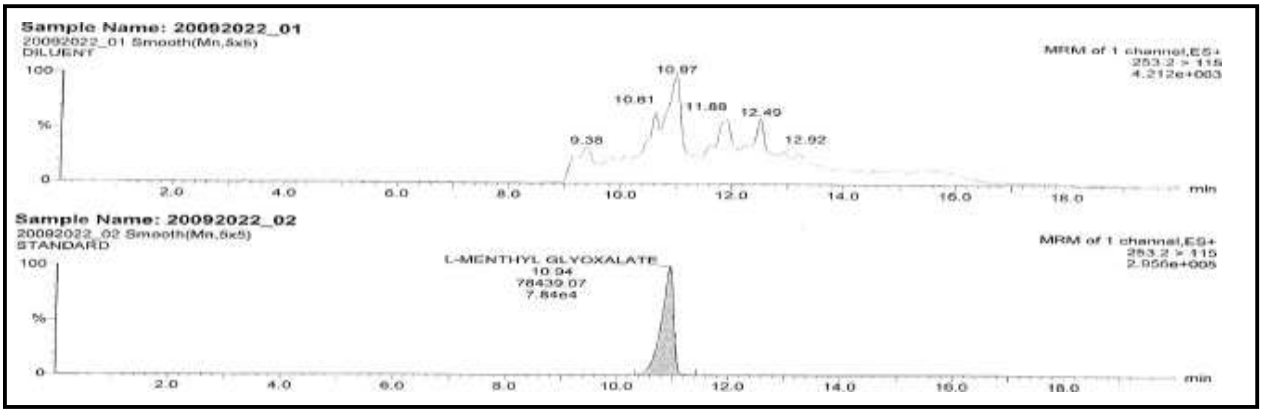


Figure 3: Specimen Chromatogram for Blank and 2-Methoxy -4-amino-5-Fluoro pyrimidine

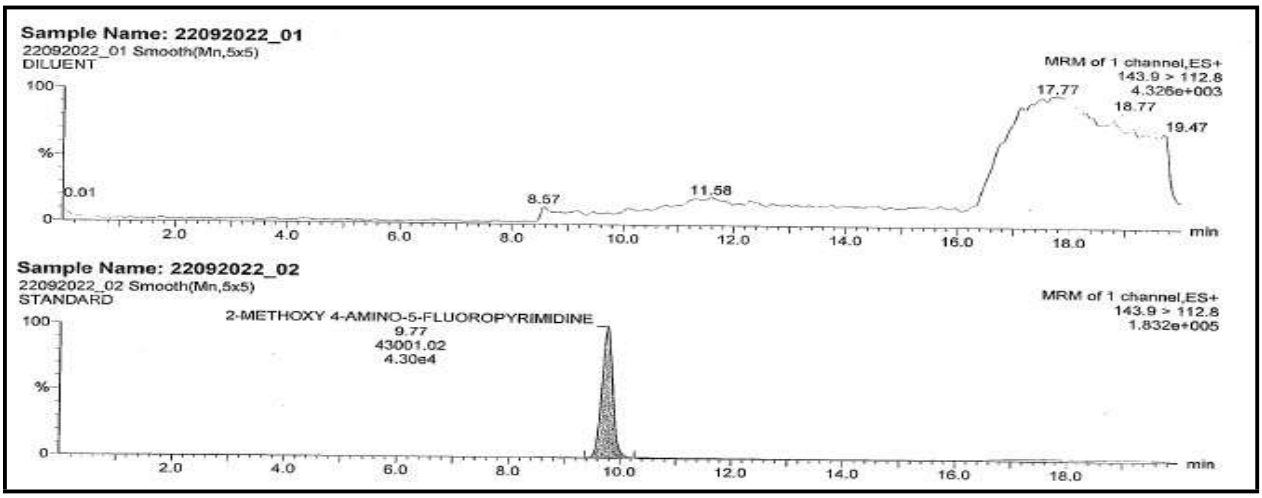
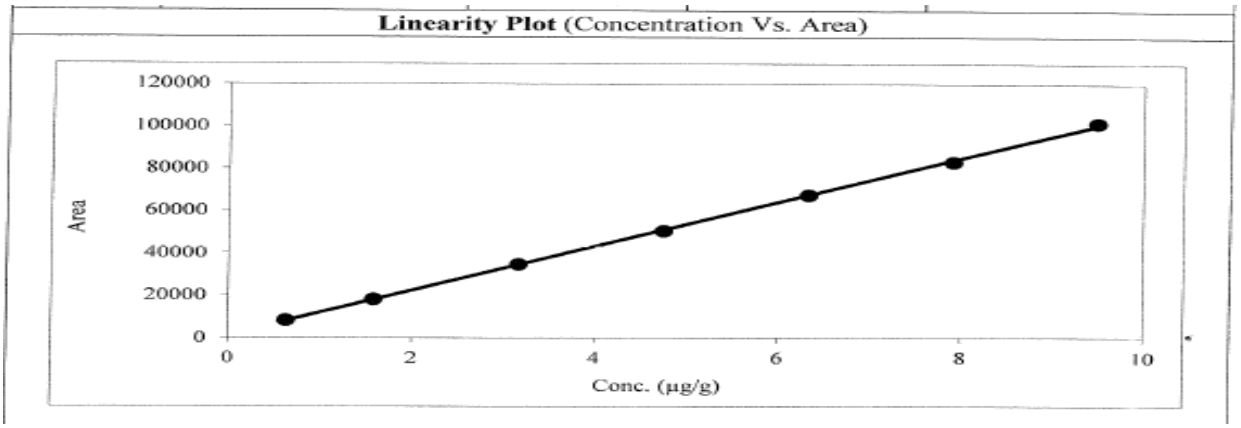


Figure 4: Linearity plot of L-Menthyl Glyoxalate



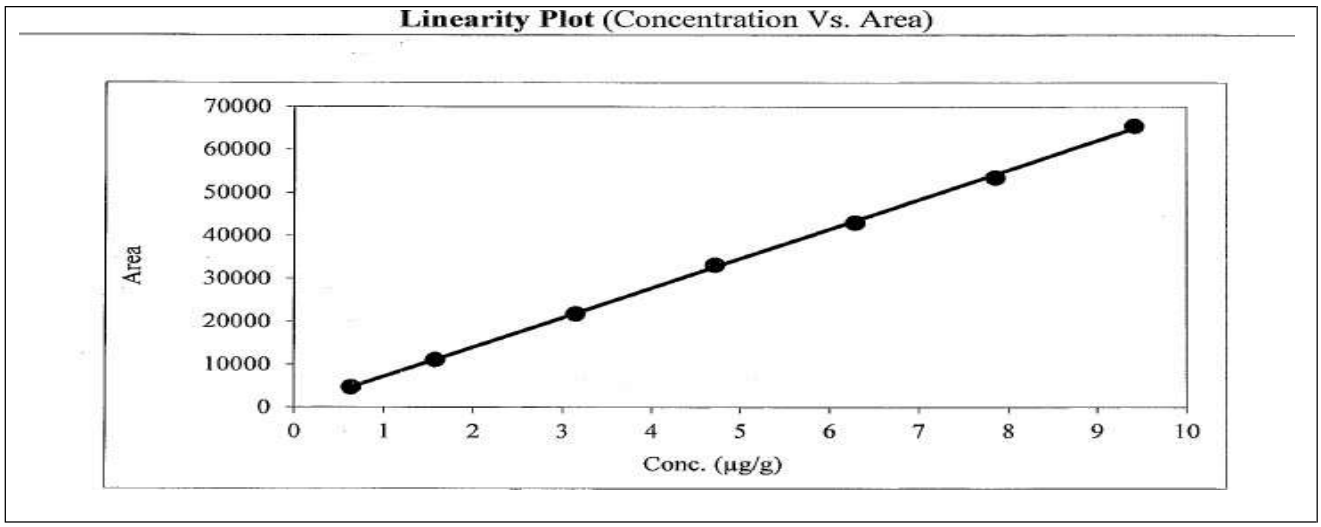


Figure 5: Linearity plot of 2-Methoxy 4-Amino-5-Fluro pyramidine

Table No 1: Chromatographic Conditions

<table>
<tr><th colspan="4">Chromatographic Conditions for LC</th></tr>
<tr><th>S.No</th><th>Parameters</th><th>L-Menthyl glyoxylate monohydrate (Method-A)</th><th>2-Methoxy 4-Amino-5-Fluro pyramidine (Method-B)</th></tr>
<tr><td>1</td><td>Mobile phase-A</td><td>Mix 1.0 mL of Formic acid 1000ml of Water</td><td>Weigh 126mg of Ammonium formate in 1000 mL of Water and add 1.0 ml Ammonia to the solution</td></tr>
<tr><td>2</td><td>Mobile phase-B</td><td>Methanol</td><td>Acetonitrile</td></tr>
<tr><td>3</td><td>Column</td><td>Acquity UPLC CSH Phenyl Hexyl 1.7 µm. (150mm x 2.1mm)</td><td>X-Select CSH C18 150x4.6mm,3.5µm</td></tr>
<tr><td>4</td><td>Flow rate</td><td>0.25mL/min</td><td>0.3mL/min</td></tr>
<tr><td>5</td><td>Injection volume</td><td>5.0 µl</td><td>5.0 µl</td></tr>
<tr><td>6</td><td>Column oven temperature</td><td>40°C</td><td>45°C</td></tr>
<tr><td>7</td><td>Auto sampler temperature</td><td>10°C</td><td>10°C</td></tr>
<tr><td>8</td><td>Run time</td><td>20 min</td><td>20 min</td></tr>
<tr><td>9</td><td>Pump mode</td><td>Gradient</td><td>Gradient</td></tr>
<tr><td></td><td></td><td><table><tr><td>Time (min)</td><td>Mobile Phase A</td><td>Mobile Phase B</td></tr><tr><td></td><td></td><td></td></tr></table></td><td><table><tr><td>Time (min)</td><td>Mobile Phase A</td><td>Mobile Phase B</td></tr></table></td></tr>
</table>

					$T_{0.01}$	85	15
		$T_{0.}$	90	10	$T_{2.0}$	85	15
		$T_{0.}$	90	10	$T_{5.0}$	65	35
		T_{10}	10	90	$T_{10.0}$	65	35
		T_{13}	10	90	$T_{12.0}$	10	90
		T_{13}	90	10	$T_{14.0}$	10	90
		T_{20}	90	10	$T_{14.1}$	85	15
					$T_{20.0}$	85	15

Table No 2: List of Chemicals, Reagents, Chemicals, Sample, Standards and Impurities

S.No	Name of the materials		Grade
1	LMHG	MAFP	
2	Formic acid	Formic acid	LCMS
3	Methanol	Ammonia	LCMS
4	Sodium hydroxide	Ammonium Formate	AR &LCMS
5	Water	Water	Milli-Q
6	L-Menthyl glyoxylate monohydrate		NA
7	2-Methoxy 4-Amino-5-Fluro pyramidine		NA
8	Salicylic acid		NA
9	Emtricitabine coupled ester		NA
10	Emtricitabine sulfoxide		NA
11	Emtricitabine acid		NA
12	Desfluoro Emtricitabine		NA
13	5-Fluorouracil Analog		NA
14	Emtricitabine		NA

Table No 3: Results for system precision

Sample Preparation ID	Area of L-Menthyl Glyoxalate	Area of 2-Methoxy 4-Amino 5-Fluoropyrimidine
1	78439	39706
2	80464	37454
3	78524	38826
4	79475	38255
5	76820	36404
6	77051	37046
Mean	78462	37949

SD	1394.68	1216.33
%RSD	1.8	3.2

Table No 4a: Results for Identification

Impurity Name	**Impurity Stock Solution (RT in minutes)**	**Spiked Sample Solution (RT in minutes)**
L-Methyl Glyoxalate monohydrate	10.94	11.01
2-Methoxy 4-Amino-5-Fluro pyramidine	9.77	9.71

Table No 4b: Results for Specificity

Impurity Name	**Area**		**MRM Trace**
	Control Sample	**Spiked Sample**	
L-Methyl Glyoxalate monohydrate	ND	44077	253.2 → 115.0
2-Methoxy4-Amino-5-Fluro pyramidine	ND	36770	143.9 → 112.8

Table No 5: Results for Limit of Quantitation and Limit of detection

Impurity Name	**LOQ Concentration (µg/g)**	**USP S/N**	**LOD Concentration (µg/g)**	**USP S/N**
L-Menthyl Glyoxalate	0.63	222.7	0.32	82.6
2-Methoxy 4-Amino-5-Fluro pyramidine	0.63	298.8	31	171.8

Table No 6: Results for Precision at LOD & LOQ Level

Injection ID	**Area of L-Menthyl Glyoxalate**		**Area of 2-Methoxy 4-Amino-5-Fluro pyramidine**	
	LOD	**LOQ**	**LOD**	**LOQ**
1	5308	8329	2818	4750
2	5271	8147	2793	4716
3	5311	8235	2771	4712
4	5239	8245	2713	4701

5	5271	8153	2763	4706
6	5255	8134	2744	4804
Mean	5276	8207	2767	4732
SD	26.67	76.1	36.75	39.5
%RSD	0.5	0.9	1.3	0.63

Table No 6a: Results for Accuracy LOQ Level for L-Menthyl Glyoxalate

Sample Preparation ID	Area of Spiked Sample (L-Menthyl Glyoxalate)	Area of Neat Standard (L-Menthyl Glyoxalate)	% Recovery
LOQ Accuracy-1	8237	8284	100.0
LOQ Accuracy-2	8284	8171	100.7
LOQ Accuracy-3	8272	8257	100.4

Table No 6a: Results for Accuracy LOQ Level for2-Methoxy 4-Amino-5-Fluro pyramidine

Sample Preparation ID	Area of Spiked Sample (2-Methoxy 4-Amino-5-Fluro pyramidine)	Area of Neat Standard (2-Methoxy 4-Amino-5-Fluro pyramidine)	% Recovery
LOQ Accuracy-1	4992	4768	105.3
LOQ Accuracy-2	4994	4822	105.3
LOQ Accuracy-3	4904	4633	103.4

Table No 7a: Results for Linearity of L-Menthyl Glyoxalate

L-Menthyl Glyoxalate		
% Level	**Concentration (µg/g)**	**Area**
LOQ	0.63	8179
25	1.58	18135
50	3.16	34542
75	4.75	50457
100	6.33	67243
125	7.91	82933
150	9.49	101450
Slope	10426	

Intercept	1429.942
Correlation Coefficient	0.9998

Table No 7b: Results for Linearity of L-Menthyl Glyoxalate

2-Methoxy 4-Amino-5-Fluro pyramidine		
% Level	**Concentration (µg/g)**	**Area**
LOQ	0.63	4736
25	1.57	11110
50	3,14	21872
75	4.71	33167
100	6.28	43059
125	7.85	53542
150	9.41	65466
Slope	6857	
Intercept	376.77	
Correlation Coefficient	0.9998	

Table No 8: Results for Method precision

Sample Preparation ID	**Area of L-Menthyl Glyoxalate**	**Area of 2-Methoxy 4-Amino-5-Fluro pyramidine**
1	71543	45275
2	72164	44776
3	71999	45304
4	70869	45001
5	71076	45341
6	71901	45270
Mean	71592	45161
SD	525.3	224.3
%RSD	0.7	0.5

Table No 9: Results for Accuracy (µg/g)

Preparation ID	Added of L-Menthyl Glyoxalate in µg/g	Found of L-Menthyl Glyoxalate in µg/g	Added of 2-Methoxy 4-Amino-5-Fluro pyramidine in µg/g	Found of 2-Methoxy 4-Amino-5-Fluro pyramidine in µg/g
1	6.33	5.77	6.28	6.68
2	6.33	5.82	6.28	6.61
3	6.33	5.81	6.28	6.69
4	6.33	5.72	6.28	6.64
5	6.33	5.73	6.28	6.69
6	6.33	5.80	6.28	6.68
	Mean	5.78	**Mean**	6.67
	SD	0.04	**SD**	0.03
	%RSD	0.7	**%RSD**	0.4

Table No 10a: Results for % Recovery of L-Menthyl Glyoxalate

Sample Preparation ID	Area of Spiked Sample (L-Menthyl Glyoxalate)	Area of Neat Standard (L-Menthyl Glyoxalate)	% Recovery
100% Accuracy-1	69244	69451	100.7
100% Accuracy-2	69438	68026	100.9
100% Accuracy-3	69809	68883	101.5
150% Accuracy-1	100660	100096	100.2
150% Accuracy-2	99903	100545	99.5
150% Accuracy-3	102057	100656	101.6

Table No 10b: Results for % Recovery of L-Menthyl Glyoxalate

Sample Preparation ID	Area of Spiked Sample (2-Methoxy 4-Amino-5-Fluro pyramidine)	Area of Neat Standard (2-Methoxy 4-Amino-5-Fluro pyramidine)	% Recovery
100% Accuracy-1	44497	42879	104.8
100% Accuracy-2	44288	42452	104.8
100% Accuracy-3	43803	42029	103.2
150% Accuracy-1	63279	64281	99.7
150% Accuracy-2	64376	62791	101.4
150% Accuracy-3	65428	63390	103.1

Parameter	Variation	L-Menthyl Glyoxalate	
		RT (minutes)	%RSD
Flow rate	-10%	11.49	2.1
	+10%	10.76	1.2
Source cleaning	Before	10.96	0.7
	After	10.98	0.9
Parameter	**Variation**	2-Methoxy 4-Amino-5-Fluro pyramidine	
		RT (minutes)	**%RSD**
Flow rate	-10%	10.66	0.9
	+10%	8.90	9.6
Source cleaning	Before	9.77	2.9
	After	9.71	3.2

Conclusion:

Analytical test method for determination of L-Menthyl Glyoxalate and 2-Methoxy 4-Amino-5-Fluro pyramidine in Emtricitabine by LC-MS/MS was validated for System Suitability, Identification, Specificity, Linearity, Method Precision, Accuracy, Range (Linearity, Precision and Accuracy)and Robustness (Flow Rate & Source Cleaning) and meets all the pre-established acceptance criteria.

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References:

1. E. De Clercq, Biophys. Acta, 1587: 258–275 (2002).
2. J.W. Beach, Clin. Ther, 20: 2–25 (1998).
3. US Department of Health and Human Services , Guidelines for the Use of Antiretroviral Agents

in HIV-Infected Adults and Adolescents, March, (2004).
4. J.E. Gallant, J. Clin. Virology, 25: 317–333 (2002).
5. J. Darbyshire, Trop. Med. Int. Health, 5: A26–A31 (2000).
6. P. Clevenbergh, S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, H. Trout, and J.F. Bergmann. Curr. HIV Res. 2: 309–321 (2004).
7. M. Boffito, E. Acosta, D. Burger, C.V. Fletcher, C. Flexner, R. Garaffo, G. Gatti, M. Kurowski, C.F. Perno, G. Peytavin, M. Regazzi, and D. Back. Antivir. Ther. 10: 375–392 (2005).
8. Guidance for Industry – Bioanalytical Method Validation (CDER, FDA); 2001.
9. S.C. Piscitelli, and K.D. Gallicano. N. Engl. J. Med. 344: 984–996 (2001).
10. R.E. Aarnoutse, J.M. Schapiro, C.A.B. Boutcher, Y.A. Hekster, and D.M. Burger. Drugs 63: 741–753 (2003).
11. D Saxena, S Damale, A Joshi, A Datar. International Journal of Life Sciences Biotechnology and Pharma Research, 3: 196 (2014)
12. T Delahunty, L Bushman, B Robbins, CV Fletcher. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 877(20-21),1907- 1914 (2008).
13. BS Rao, S Nagaraju, BV Kiran. Asian Journal of Research in Chemistry, 6(10): 936-944 (2013).
14. PT Nagaraju, KPR Channa Basava, KPT Shantha. International Journal of Chemtech Research. 3(1):23-28 (2011).