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ANALYTICAL VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCES PRALATREXATE INJECTION 20 MG/ML BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract:
A novel, simple, sensitive and stability-indicating HPLC (high-performance liquid chromatography) method was developed and validated for the related substances of Pralatrexate Injection 20mg/Ml. Reversed-phase chromatography was performed on Agilent 1200 series with Software Empower-2 and Agilent 1200 series with PDA 1100 series Software Empower-2 photodiode array detector using Hypersil BDS C-18 (250 × 4.6) mm, 5 µm column with pH 5.3 (adjusted with sodium hydroxide) of Sodium dihydrogen phosphate monohydrate as buffer & methanol- 90&10 ratio (v/v) as mobile phase-A and buffer & methanol- 20&80 ratio (v/v) as Mobile phase-B at a flow rate of 1.0 mL/min. Gradient profile at Initial: 95-5, 18 minutes: 70-30, 30 minutes: 67-33, 50 minutes: 39-61, 51minutes: 95-5, 60 minutes: 95-5 and with UV detection at 242 nm. The method is simple, specific, precise and accurate for the determination of related substances of Pralatrexate Injection 20mg/mL

1. Summary

Table 1Summary of Results

Validation parameter	Procedure	Acceptance criteria	Results	Status
• Specificity	Inject blank, placebo solution, sample solution and spiked sample solutions	There should not be any interference from the blank and placebo at the retention time of Pralatrexate peak and known impurities peak.	No interference was observed from blank and placebo at the retention time of Pralatrexate and known impurities peak.	Conforms
	Individual impurity solutions to be injected	All known impurity peaks should be separated from each other and retention time of each known impurity peak in individual standard solution chromatogram should match with retention time of	All known impurity peaks are found to be separated from each other and retention time of each known impurity peak in individual standard solution chromatogram is matching with retention time of the individual peak in the spiked sample solution chromatogram.	Conforms

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		the individual peaks in the spiked sample solution chromatogram. The purity angle should be less than purity threshold for Pralatrexate peak in the spiked sample with all known impurities peak of Pralatrexate	The purity angle is found to be less than purity threshold for Pralatrexate peak in the spiked sample with all known impurities peak of Pralatrexate	Conforms
• Force Degradation	Performed acid, base, oxidative, thermal and photolytic degradation	The purity angle of Pralatrexate peak and known impurities peak should be less than purity threshold under all stress conditions.	The purity angle is found to be less than purity threshold for Pralatrexate peak and observed known impurities peak under all stress condition	Conforms
• Linearity	n = 1	Pralatrexate r ≥ 0.990 Hydro-PLT r ≥ 0.990 Pt-MADEC r ≥ 0.990	Pralatrexate r = 0.99999 Hydro-PLT r = 1.0 Pt-MADEC r = 1.0	Conforms
• Accuracy	n = 3 4 concentrations	80.0 % - 120.0 %	Hydro-PLT 106.07% to 108.91% Pt-MADEC 99.76% to 110.03%	Conforms
• Range	To be concluded from Precision, Linearity and Accuracy data	Concluded from Precision, Linearity and Accuracy data	The method has a range in between QL to 150% of specification limit, which is established from the data analysis of linearity, precision and accuracy studies.	Conforms
• Precision				

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System precision Repeatability (Method Precision)	Replicate Injections of standard Preparation =6 n = 6	%RSD of six replicate standard injections should not be more than 10.0. CV ≤ 10.0	CV = 0.20% Hydro-PLT CV = 0.0% Pt-MADEC CV = 0.0% Total Impurities CV=0.12%	Conforms Conforms
Intermediate precision	n = 12	CV ≤ 10.0	Hydro-PLT CV = 1.20% Pt-MADEC CV = 1.46% Total Impurities CV=0.65%	Conforms
• Detection limit		The RSD of area counts for limit of detection should not be more than 33%	**Detection Limit (%w/w)** Hydro-PLT : 0.016 Pt-MADEC : 0.010 Pralatrexate :0.004 **Verification at DL (%RSD)** Hydro-PLT : 15.30 Pt-MADEC : 14.77 Pralatrexate : 13.74	Conforms
• Quantitation limit		The RSD of area counts for limit of Quantitation should not be more than 10%	**Quantitation Limit (%w/w)** Hydro-PLT :0.033 Pt-MADEC:0.019 Pralatrexate:0.010 **Precision at QL (%RSD)** Hydro-PLT :0.93 Pt-MADEC :1.13 Pralatrexate :4.85	Conforms
• Stability of analytical solution				
Stability of	Monitoring the area	Cumulative %	The standard solution was	Conforms

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analytical solution (25°C)	of Pralatrexate peak, Known impurities peak and unknown impurities peak	RSD ≤ 10.0	found to be stable up to 60 hours with cumulative RSD of 0.80% The sample solution was found to be stable up to 51 hours with highest cumulative RSD of 1.08% for Hydro-PLT peak, 0.89 for Pt-MADEC and 0.95 for highest unknown The spike sample solution was found to be stable up to 49 hours with highest cumulative RSD of 0.26 % for Hydro-PLT peak, 0.47 for Pt-MADEC and 0.81 for highest unknown.	
Stability of analytical solution(5°C)	Monitoring the area of Pralatrexate peak, Known impurities peak and unknown impurities peak	Cumulative % RSD ≤ 10.0	The standard solution was found to be stable up to 80 hours with cumulative RSD of 0.37% The sample solution was found to be stable up to 71 hours with highest cumulative RSD of 0.16% for Hydro-PLT peak, 2.64 for Pt-MADEC and 0.59 for highest unknown The spike sample solution was found to be stable up to 69 hours with highest cumulative RSD of 0.14 % for Hydro-PLT peak, 0.23 for Pt-MADEC and 0.43 for highest unknown.	Conforms

Conclusión

The results of this validation confirm that the method used is suitable for the determination of Related Substances of Pralatrexate Injection 20 mg/mL within the investigated range and analytical concentration

2. Procedure

Reagents:

Water (HPLC grade)
Acetonitrile (HPLC grade)
Methanol (HPLC grade)
Sodium dihydrogen phosphate monohydrate **(**AR grade)
Dimethyl sulphoxide (DMSO) (GC Grade)
Sodium Hydroxide **(**AR grade)
Hydrochloric acid (AR grade)

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Preparation of Buffer Solution
Weigh accurately about 5.48g of Sodium dihydrogen phosphate monohydrate into 2 L bottle. Add 2 L of HPLC grade water. Mix well using a magnetic stir bar until the material is completely dissolved, adjust the pH to 5.3 ± 0.05 with diluted Sodium Hydroxide. Filter the solution through a 0.45 µm nylon membrane filter and degas it well.

Preparation of Mobile phase-A
Mix 900 mL of buffer solution and 100mL of methanol into a 1 L bottle and mix it well.

Preparation of Mobile phase-B
Mix 200mL of buffer solution and 800mL of methanol into a 1 L bottle and mix it well.

Preparation of diluents
Mix 900mL of Buffer solution and 100mL of Acetonitrile into a 1 L bottle and mix well and Degas by sonication.

Preparation of Blank
Transfer 2mL of DMSO into 20mL volumetric flask and make up to the volume with diluent.

Chromatographic conditions:

Column — Hypersil BDS C-18 (250 × 4.6) mm, 5 µm.

Flow Rate — 1.0 mL/min.

Gradient Program

Time (min.)	MP-A%	MP-B%
Initial	95	5
18	70	30
30	67	33
50	39	61
51	95	5
60	95	5

Column Temperature — 25° C

Sample Temperature — 5°C

Detector Wavelength — 242 nm

Injection Volume — 10 µL

Run Time — 60 minutes

Preparation of 1N Sodium hydroxide solution
Weigh about 4 g of Sodium hydroxide into a 100 mL volumetric flask, add 50 mL of HPLC grade water and sonicate to dissolve, dilute to volume with HPLC grade water and sonicate to dissolve.

Preparation of 1N Hydrochloric acid solution
Add 50 mL of HPLC grade water into a 100 mL volumetric flask, accurately transfer 8.7mL of Hydrochloric acid into it, dilute to volume with HPLC grade water and mix well.

Preparation of System suitability solution

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Weigh about 5 mg of Pralatrexate reference/working standard into a 10 mL volumetric flask, add 1mLof DMSO and sonicate to dissolve. Add 1mL of 1N Sodium hydroxide and keep it in a water bath at 30°c for 15 minutes. Add 1mL of 1N Hydrochloric acid and finally make up to the volume with diluent.

Preparation of Standard solution

Weigh about 10 mg of Pralatrexate reference/working standard into a 50 mL volumetric flask, add 5 mL of DMSO and sonicate to dissolve completely then dilute to volume with diluent and shake well. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluent and shake well. to get a solution having a known concentration of about 2 ppm.

Evaluation of system suitability parameters

Before proceeding for the system suitability evaluation, equilibrate the column sufficiently to get a stable baseline. Inject the blank, system suitability solution and diluted standard solution into the chromatograph and record the chromatograms.

- In the chromatogram obtained with system suitability solution, the number of theoretical plates for Pralatrexate peak should not be less than 15000
- The resolution between Pralatrexate Amide and Pralatrexate should not be less than 8.0
- The relative standard deviation of area counts of six injections of standard solution should not be more than 5.0%

Retention time of Pralatrexate is about 18 minutes and the relative retention time (RRT) of relative compounds with respect to Pralatrexate is listed below:

Related substances	Relative retention time (about)	RF	DL(%)	QL(%)
Hydro-PLT	0.77	1.21	0.016	0.033
Pt-MADEC	1.31	1.00	0.010	0.019
Pralatrexate	-	-	0.004	0.010

- Apart from the known related substances, some known process impurities of Pralatrexate API as given in the table below. Disregard known process impurities peaks in related substances calculations.

Related substances	Relative retention time (about)
PLt-Diglu-A_Isomer A	14.40
PLt-Diglu-A_Isomer B	14.73
PLt-Diglu-B	16.50

Preparation of sample solution (for 2mL vial)

Transfer 1mL of sample solution into a 20mL volumetric flask and add 2 mL of DMSO mix and dilute up to the mark with diluent.

Preparation of Placebo solution (for 2mL vial)

Transfer 1 mL of placebo into a 20 mL volumetric flask and add 2 mL of DMSO mix and dilute up to the mark with diluent.

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Procedure

Inject the sample solution and placebo solution into the chromatograph and record the chromatogram. Disregard the peak in the sample chromatogram which is observed at the same retention time in the blank and placebo chromatogram. Disregard the peak of Process impurities PLt-Diglu-A_Isomer-1, PLt-Diglu-A_Isomer-2 and PLt-Diglu-B in the sample Chromatogram.

Injection Sequence:

Name of the Injection	No. of Injections
Blank	1
System suitability	1
Blank	1
Standard solution	6
Placebo solution	1
Sample solution	1
System suitability	1

Reporting and Calculation

Report all the known impurities, unknown individual impurities, and total impurities

Calculate the unknown impurities in % w/w by using the following formula

$$\text{Individual Impurity} = \frac{A_T}{A_S} \times \frac{W_S}{50} \times \frac{1}{100} \times \frac{20}{1} \times \frac{P}{L}$$

Calculate the known impurities in % w/w by using the following formula

$$\text{Individual Impurity} = \frac{A_T}{A_S} \times \frac{W_S}{50} \times \frac{1}{100} \times \frac{20}{1} \times \frac{P}{L} \times \text{RF}$$

Total Impurities = Sum of all known and unknown impurities.

Where,

A_T	=	Area count of Individual impurity peak in the chromatogram obtained with sample solution.
As	=	Average area count of Pralatrexate peak in the chromatogram obtained with Standard solution.
Ws	=	Weight of Pralatrexate working/reference standard taken in mg.
L	=	Label claim (mg/mL).
P	=	Potency of Pralatrexate working/reference standard in % w/w.
RF	=	Response factor

3. Specificity

The specificity was established by injecting blank, placebo, sample, spiked sample and individual impurity solutions into the system as per methodology. The results are as follows:

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Table 2Retention time of impurities in spiked sample

Component	Retention time (Spiked Sample)	Retention time (Individual stock)	Rel. Retention time (Spiked Sample)	Purity Angle (Spiked Sample)	Purity Threshold (Spiked Sample)
Hydro-PLT	14.13	14.06	0.77	0.471	0.741
Pt-MADEC	24.09	24.08	1.31	1.031	1.499
*PLT-Diglu-A-Isomer-1	14.49	14.40	0.79	7.343	9.958
*PLT-Diglu-A-Isomer-2	14.82	14.73	0.80	6.087	8.279
PLT-Diglu B	16.59	16.50	0.90	1.939	2.608
Pralatrexate	18.44	18.44	1.00	0.151	0.285

***- Two peak observed of PLT –Diglu-A in this method (no Individual standard of Isomer-1 and Isomer-2)**

Conclusion
No interference was observed from blank, placebo at retention time of known impurities and Pralatrexate peak.

The purity angle is found to be less than purity threshold for Pralatrexate peak in the spiked sample with all known impurities peak of Pralatrexate**.**

All known impurity peaks are found to be separated from each other and retention time of each known impurity peak in individual standard solution chromatogram is matching with retention time of the individual peaks in the spiked sample solution chromatogram.

4. Force Degradation

Force degradation is performed by subjecting the sample for different stress conditions like base, acid, oxidative, thermal and photolytic stress for different length of time. Samples were analyzed to evaluate degradation profile and purity factor of Pralatrexate and known impurities peak using DAD.

Base stress
Pralatrexate Injection was subjected to base stress by treating with 2mL of 5N Sodium hydroxide for 3hrs at room temperature.

Acid stress
Pralatrexate was subjected to acid stress by treating with 2mL of 5N Hydrochloric acid for 4hrs at room temperature.

Oxidative stress
Pralatrexate Injection was subjected to oxidative stress by treating with 3 mL of 30% Hydrogen peroxide for 3hrs at room temperature.

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Thermal stress
Pralatrexate Injection was subjected to thermal stress by keeping at 105°C ± 5°C for about 7 hours, followed by analysis as per methodology.

Photolytic stress
Pralatrexate injection sample was subjected to photolytic stress for light intensity of total 1.2 million lux hours and near UV Fluorescent light (total 200 WH/m^2).

Table 3 Degradation study

Sample	Hydro- PLT	Pt-MADEC	Total Unknown	Total Impurities	Total Degradation
Control	0.10	0.05	0.15	0.30	-
Acid Treated (5N 2ml 4 hrs at RT)	1.47	0.06	0.17	1.70	1.40
Alkali Treated (5N 2ml 3 hrs at RT)	11.76	0.06	0.10	11.92	11.62
Peroxide Sample(3ml of 30% H2O2 3 Hrs at RT)	0.12	0.08	0.20	0.40	0.02
Thermal Sample(7 Hrs at 105°C)	6.88	0.03	0.41	7.32	6.94
Photolytic Sample (1.2 Million LUX)	0.21	0.14	5.02	5.37	4.99

Table 4 Peak purity

Sample	Hydro-PLT		Pt- MADEC		Pralatrexate	
	Peak angle	Peak Threshold	Peak angle	Peak Threshold	Purity angle	Purity Threshold
Control	2.739	3.195	4.196	4.701	0.057	0.248
Acid Treated (5N 2ml 4 hrs at RT)	0.283	0.522	6.247	7.822	0.104	0.278
Alkali Treated (5N 2ml 3 hrs at RT)	0.07	0.283	6.612	8.484	0.096	0.281
Peroxide Sample(3ml of 30% H2O2 3 Hrs at RT)	2.631	3.017	4.304	4.845	0.062	0.251
Thermal Sample(7 Hrs at 105°C)	0.315	0.326	9.512	21.225	0.058	0.250
Photolytic Sample (1.2 Million LUX)	3.574	3.623	3.567	7.553	0.075	0.256

Conclusion:
The purity angle is found to be less than purity threshold for Pralatrexate peak and observed known impurities peak in all the stress conditions of Pralatrexate Injection.

5. Linearity

The linearity was determined by measurement of µg/mL concentrations of Pralatrexate, Hydro-PLT, Pt-MADEC, from QL to 150 % of specification.

The respective concentrations were plotted against corresponding area counts to draw linearity graph of Pralatrexate, Hydro- PLT, and Pt-MADEC, Correlation coefficient was calculated. The results are as follows

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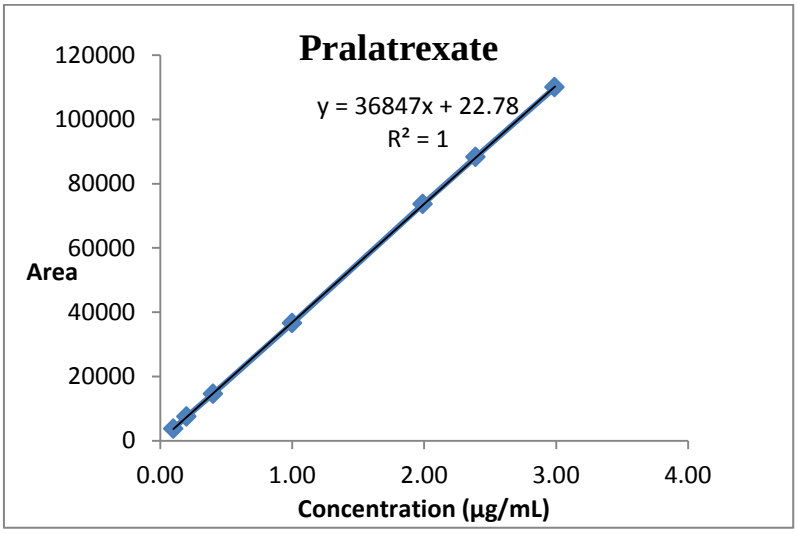
Table 5 Results of the Linearity

Pralatrexate

c[µg/mL]	Area count
2.99	109970
2.39	88273
1.99	73625
1.00	36574
0.40	14627
0.20	7530
0.10	3765

The resulting regression line was calculated in accordance with the equation: y = a * x + b



r =	0.99999
a =	36847.24807
b =	22.78000
ss_{res} =	289611

Hydro-PLT

c[µg/mL]	Area count
14.79	451839
11.83	362572
9.86	302067
4.93	150047
1.97	60547
0.99	30815
0.49	15450
0.39	12110
0.20	6014

The resulting regression line was calculated in accordance with the equation: y = a * x + b

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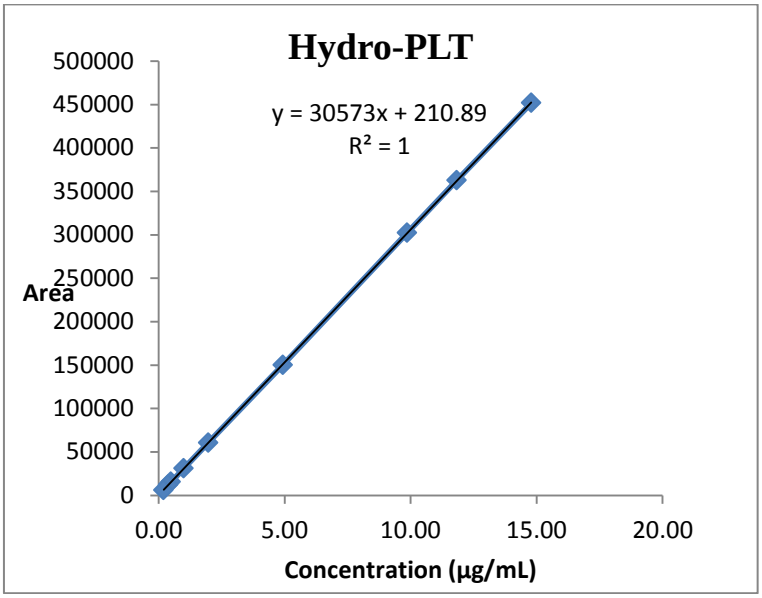
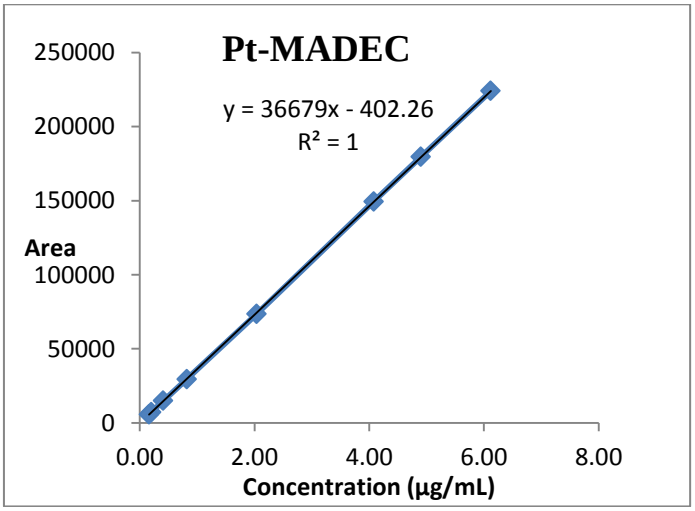


r =	1.0
a =	30573.44241
b =	210.89361
ss_{res} =	2008288

Pt-MADEC

c[µg/mL]	Area count
6.12	224046
4.90	179598
4.08	149388
2.04	73570
0.82	29460
0.41	14867
0.20	7240
0.16	5612

The resulting regression line was calculated in accordance with the equation: y = a * x + b



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r =	1.0
a =	36679.07570
b =	-402.26099
ss_{res} =	1037060

Where; r = Correlation coefficient
a = Slope
b = Intercept
ss_{res} = Sum of Residue square
y = area
x = concentration

Conclusion
The method is linear over specified range from QL to 150% of specification of known impurities.

6. Range

The method has a range in between QL to 150% of specification limit, which is established from the data analysis of linearity, precision and accuracy studies.

7. Accuracy

The accuracy was determined by assaying known amount of impurity standard at QL, 50%, 100% and 150% of specification Level of known impurities.
A 3-fold measurement at [QL], [50%], [100%] and [150%] respectively (12 in total) was carried out.

Table 6 Results of Accuracy for Hydro-PLT

Hydro-PLT				
Level	**Sample**	**Amount added (mg)**	**Amount recovered (mg)**	**% Recovery**
QL -1	1	0.00597	0.00638	106.87
QL -2	2	0.00597	0.00647	108.38
QL -3	3	0.00597	0.00650	108.88
50% -1	1	0.09881	0.10659	107.87
50% -2	2	0.09881	0.10761	108.91
50% -3	3	0.09881	0.10630	107.58
100% - 1	1	0.19761	0.21002	106.28
100% - 2	2	0.19761	0.20996	106.25
100% - 3	3	0.19761	0.20960	106.07
150% - 1	1	0.29642	0.31610	106.64
150% - 2	2	0.29642	0.31618	106.67
150% - 3	3	0.29642	0.31642	106.75
			Overall mean	**107.26**
			Overall SD	**0.885**
			Overall % RSD	**0.83**

Table 7 Results of Accuracy for Pt-MADEC

Pt-MADEC				
Level	**Sample**	**Amount added (mg)**	**Amount recovered (mg)**	**% Recovery**
QL -1	1	0.00424	0.00432	101.89

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QL -2	2	0.00424	0.00423	99.76
QL -3	3	0.00424	0.00434	102.36
50% -1	1	0.04075	0.04404	108.07
50% -2	2	0.04075	0.04438	108.91
50% -3	3	0.04075	0.04403	108.05
100% - 1	1	0.08150	0.08725	107.06
100% - 2	2	0.08150	0.08692	106.65
100% - 3	3	0.08150	0.08710	106.87
150% - 1	1	0.12224	0.13385	109.50
150% - 2	2	0.12224	0.13423	109.81
150% - 3	3	0.12224	0.13450	110.03
			Overall mean	**106.58**
			Overall SD	**2.622**
			Overall % RSD	**2.46**

Conclusion:
The recovery was found within 80.0% to 120.0% at each level, hence method is accurate.

8. Precision

8.1 Repeatability (System precision and Method precision)

The repeatability was determined by a 6-fold measurement of sample with independent sample preparation at 100% level and analysing as per methodology. It was also established by injecting standard six times into the chromatographic system. The average (x), standard deviation (s), %RSD (CV) and 95% confidence interval (CI) was calculated. The results are tabulated below:

Table 8 Results of System precision

Injection	Peak area of Pralatrexate
1	73411
2	73399
3	73366
4	73123
5	73543
6	73491
x =	73389
s =	145.59453
CV =	0.20

Table 9 Results of Repeatability (Method precision)

Sample Preparation	%Hydro-PLT	% Pt-MADEC	% Highest unknown impurity	%Total Impurity
1	1.16	0.49	0.07	3.37
2	1.16	0.49	0.07	3.37
3	1.16	0.49	0.07	3.37
4	1.16	0.49	0.07	3.37
5	1.16	0.49	0.07	3.37
6	1.16	0.49	0.08	3.38
x =	1.16	0.49	0.07	3.37

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s =	0.000	0.000	0.004	0.004
CV =	0.00	0.00	5.71	0.12
CI =	0.00	0.00	0.00	0.00

8.2 Intermediate precision

The intermediate precision was determined by 6-fold measurement of sample with independent sample preparation at 100% level and analysing as per methodology, by a different analyst, on a different day, using column of different serial no. and calculating the same statistical parameters as that of repeatability. The results are tabulated below:

Table 10 Results of Intermediate precision

Sample Preparation	%Hydro-PLT	% Pt-MADEC	% Highest unknown impurity	%Total Impurity
1	1.18	0.48	0.08	3.40
2	1.18	0.48	0.08	3.40
3	1.19	0.48	0.08	3.42
4	1.19	0.48	0.08	3.42
5	1.19	0.48	0.08	3.42
6	1.18	0.47	0.08	3.38

x =	1.19	0.48	0.08	3.41
s =	0.005	0.004	0.000	0.016
CV =	0.42	0.83	0.00	0.47
CI =	0.01	0.00	0.00	0.02

Table 12 Results Comparison of Precision study for Hydro-PLT and Pt-MADEC

Sample Preparation	% Hydro-PLT		% MADEC	
	Method Precision	Intermediate precision	Method Precision	Intermediate precision
1	1.16	1.18	0.49	0.48
2	1.16	1.18	0.49	0.48
3	1.16	1.19	0.49	0.48
4	1.16	1.19	0.49	0.48
5	1.16	1.19	0.49	0.48
6	1.16	1.18	0.49	0.47
x =	**1.17**		**0.48**	
s =	**0.014**		**0.007**	
CV =	**1.20**		**1.46**	
CI =	**0.01**		**0.01**	

Table 13 Results Comparison of Precision study for Highest unknown impurity and total Impurities:

Sample Preparation	% Highest unknown impurity		% Total Impurities	
	Method Precision	Intermediate precision	Method Precision	Intermediate precision
1	0.07	0.08	3.37	3.40
2	0.07	0.08	3.37	3.40
3	0.07	0.08	3.37	3.42
4	0.07	0.08	3.37	3.42
5	0.07	0.08	3.37	3.42
6	0.08	0.08	3.38	3.38
x =	**0.08**		**3.39**	

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s =	**0.005**	**0.022**
CV =	**6.25**	**0.65**
CI =	**0.01**	**0.02**

Conclusion

The method has an acceptable level of System precision, Method Precision and Intermediate precision.

9. Detection Limit

The Detection Limit (DL) values of known impurities and that of Pralatrexate were determined based on calibration curve plotted between concentration of impurity and their respective responses. The respective DL of impurities were calculated from the residual standard deviation obtained from calibration curve.

$$DL = \frac{3.3\,\sigma}{S}$$

Where:

σ = the standard deviation of the response

S = the slope of the calibration curve

DL = Detection limit

Table 14 Detection Limit

Component	DL %w/w
Hydro-PLT	0.016
Pt-MADEC	0.010
Pralatrexate	0.004

Table 15 Results of Verification of Detection Limit

Verification of detection limit was performed. The results are tabulated as follows:

Injection	Area of Hydro-PLT	Area of Pt-MADEC	Area of Pralatrexate
1	4884	3169	1594
2	4633	3105	1406
3	4231	2817	1240
4	3999	2787	1160
5	6077	4059	1160
6	4653	3018	1504
Mean	**4746**	**3159**	**1344**
SD	**725.983**	**466.529**	**184.647**
%RSD	**15.30**	**14.77**	**13.74**

10. Quantitation Limit

The Quantitation Limit (QL) values of known impurities and that of Pralatrexate were determined based on calibration curve plotted between concentration of impurity and their respective responses. The respective QL of impurities were calculated from the residual standard deviation obtained from calibration curve.

$$QL = \frac{10\,\sigma}{S}$$

Where:

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σ = the standard deviation of the response
S = the slope of the calibration curve
QL = Quantitation limit

Table 16 Quantitation Limit

Component	QL %w/w
Hydro-PLT	0.033
Pt-MADEC	0.019
Pralatrexate	0.010

Table 17 Results of Precision at Quantitation Limit

Precision at Quantitation limit was performed. The results are tabulated as follows:

Injection	Area of Hydro-PLT	Area of Pt-MADEC	Area of Pralatrexate
1	9977	6876	7993
2	9914	6830	8733
3	10087	6943	8402
4	10003	6865	8264
5	9978	6805	8849
6	10173	6713	9121
Mean	**10022**	**6839**	**8560**
SD	**92.749**	**77.425**	**415.268**
%RSD	**0.93**	**1.13**	**4.85**

11. Robustness

The robustness of the HPLC method for the determination of related substances in Pralatrexate 20 mg/mL was established by varying analytical conditions one at a time from test method. System suitability parameters were monitored. The results are tabulated as follows:

Table 18 Results of the Robustness:

Change in column of different serial number

Parameters	Acceptance criteria	Results	
		Column -1	Column -2
*Resolution	Should not be less than 8.0	14.10	14.40
Theoretical plate	Should not be less than 15000	61271	54910
% RSD of six replicate standard injections	Should not be more than 5.0	0.20	0.13

Change in Flow rate by ± 0.1mL/min

Parameters	Acceptance criteria	Results	
		0.9 mL/min	1.1mL/min

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*Resolution	Should not be less than 8.0	14.95	14.68
Theoretical plate	Should not be less than 15000	55350	47614
% RSD of six replicate standard injections	Should not be more than 5.0	0.11	0.21

Change in Wavelength of detection by ± 3 nm

Parameters	**Acceptance criteria**	**Results**	
		239nm	**245nm**
*Resolution	Should not be less than 8.0	13.6	13.60
Theoretical plate	Should not be less than 15000	55975	55725
% RSD of six replicate standard injections	Should not be more than 5.0	1.31	0.86

Change in column oven temperature by ± 5°C:

Parameters	**Acceptance criteria**	**Results**	
		20°C	**30°C**
*Resolution	Should not be less than 8.0	13.55	14.70
Theoretical plate	Should not be less than 15000	54932	48459
% RSD of six replicate standard injections	Should not be more than 5.0	0.19	0.05

Change in the Initial Gradient ± 2%:

Parameters	**Acceptance criteria**	**Results**	
		- 2% absolute	**+ 2% absolute**
*Resolution	Should not be less than 8.0	14.44	14.86
Theoretical plate	Should not be less than 15000	43721	58685
% RSD of six replicate standard injections	Should not be more than 5.0	0.06	0.07

Change in pH of Mobile phase Buffer by ± 0.2 unit:

Parameters	**Acceptance criteria**	**Results**	
		pH of Buffer 5.1	**pH of Buffer 5.3**
*Resolution	Should not be less than 8.0	13.67	15.11
Theoretical plate	Should not be less than 15000	60232	39647
% RSD of six replicate standard injections	Should not be more than 5.0	0.45	0.07

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Resolution*(The Resolution between Pralatrexate and Hydro-PLT)

Conclusion
The system suitability parameters complied in every Robustness condition.

12. Stability of Analytical Solution

The stability of Standard and sample preparation as per method and spiked sample preparation was performed at 25°C and 5°C by monitoring the area counts of Pralatrexate in standard preparation and Hydro-PLT and Pt-MADEC in spiked sample preparations and observed known impurities and unknown impurities in control sample. The results are tabulated below:

Table 19 Stability of standard solution stored at (25°C)

Time point in hours	Area of Pralatrexate Peak	Cumulative RSD (%)
0	71475	-
1	71397	0.08
1	71506	0.08
3	71374	0.09
4	71715	0.19
4	71733	0.22
9	71716	0.22
15	72213	0.38
22	72157	0.43
32	72507	0.54
45	72928	0.70
53	72650	0.74
60	72915	0.80

Table 20 Results of the Solution Stability of sample stored at (25°C)

Time point in hours	Area of Hydro-PLT peak	Cumulative RSD (%)	Area of Pt-MADEC peak	Cumulative RSD (%)
0	29109	-	18536	-
6	29295	0.45	18767	0.88
13	29316	0.39	18747	0.69
23	29535	0.59	18854	0.72
35	29730	0.82	18848	0.69
44	29811	0.93	18968	0.77
51	29988	1.08	19060	0.89

Time point in hours	Highest unknown peak	Cumulative RSD (%)
0	28562	-
6	28804	0.60
13	28901	0.61
23	28945	0.59
35	28995	0.59
44	29412	0.96
51	29205	0.95

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Table 21 Results of the Solution Stability of Spiked sample stored at (25°C)

Time point in hours	Area of Hydro-PLT peak	Cumulative RSD (%)	Area of Pt-MADEC peak	Cumulative RSD (%)
0	331950	-	167946	-
4	332215	0.06	168012	0.03
11	332317	0.06	169478	0.51
21	332503	0.07	168306	0.42
33	333330	0.16	169946	0.54
41	333724	0.21	169250	0.50
49	334213	0.26	169252	0.47

Time point in hours	Highest unknown peak	Cumulative RSD (%)
0	27316	-
4	27540	0.58
11	27586	0.53
21	27630	0.51
33	27782	0.61
42	27828	0.67
48	28010	0.81

Table 22 Stability of standard solution stored at (5°C)

Time point in hours	Area of Pralatrexate Peak	Cumulative RSD (%)
0	73411	-
1	73399	0.01
2	73366	0.03
3	73123	0.19
4	73543	0.21
5	73491	0.20
9	73319	0.18
26	73929	0.32
39	73736	0.32
51	74036	0.39
61	73750	0.38
80	73793	0.37

Table 23 Results of the Solution Stability of sample stored at (5°C)

Time point in hours	Area of Hydro-PLT peak	Cumulative RSD (%)	Area of Pt-MADEC peak	Cumulative RSD (%)
0	30342	-	20687	-
11	30446	0.24	20784	0.33
17	30452	0.20	21120	1.09
29	30489	0.21	22293	3.48
42	30459	0.18	21615	3.11
52	30450	0.17	21716	2.89
71	30491	0.16	21464	2.64

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Time point in hours	Highest unknown peak	Cumulative RSD (%)
0	27210	-
11	27375	0.43
17	26918	0.85
29	27170	0.70
42	27028	0.65
52	27016	0.61
71	26969	0.59

Table 24 Results of the Solution Stability of Spiked sample stored at (5°C)

Time point in hours	Area of Hydro-PLT peak	Cumulative RSD (%)	Area of Pt-MADEC peak	Cumulative RSD (%)
0	344638	-	176789	-
9	344895	0.05	177233	0.18
15	343439	0.23	176478	0.21
27	344416	0.18	177657	0.29
40	344081	0.16	177454	0.27
50	344380	0.15	177201	0.24
69	344114	0.14	177265	0.23

Time point in hours	Highest unknown peak	Cumulative RSD (%)
0	25999	-
9	26116	0.32
15	25789	0.64
27	25998	0.52
40	25955	0.46
50	25921	0.41
69	25823	0.43

Conclusion:

The standard solution was found to be stable up to 59 hours 43 minutes with cumulative RSD of 0.8%, sample solution was found to be stable up to 50 hours 37 minutes and spiked sample solution was found to be stable up to 48 hours and 35 minutes at 25°C.

The standard solution was found to be stable up to 80 hours with cumulative RSD of 0.37%, sample solution was found to be stable up to 70 hours 55 minutes and spiked sample solution was found to be stable up to 68 hours 52 minutes at 5°C.

13. System Suitability Testing

The system suitability tests that were applied during validation exercise are as follows:

Table 25 System Suitability Tests

System suitability parameter	Acceptance criteria
Resolution	Should not be less than 8.0
Theoretical plate of Pralatrexate Peak	Should not be less than 15000
% RSD of six replicate standard injections	Should not be more than 5.0

The system suitability criteria complied throughout the Validation exercise.

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Table 26 Results of System Suitability Test

Parameter	Resolution*	Theoretical plate	%RSD
Solution stability at 5deg ,method precision ,selectivity	14.1	61271	0.2
Linearity	14.19	52317	0.25
Solution stability at 25 deg C	14.4	61650	0.22
Recovery	13.51	49964	0.07
Ruggedness, Forced degradation	14.4	56075	0.13
Recovery at LOQ level	13.6	50158	0.08
Robustness minus wavelength	13.6	55975	1.31
Robustness plus wavelength	13.6	55725	1.22
Robustness plus Flow	14.68	47614	0.21
Robustness minus Flow	14.95	55350	0.11
Robustness Plus Temperature	14.7	48459	0.05
Robustness minus Temperature	13.55	54932	0.19
Robustness Plus pH	15.11	39647	0.07
Robustness minus pH	13.67	60232	0.45

Resolution*(The Resolution between Pralatrexate peak and Hydro-PLT)

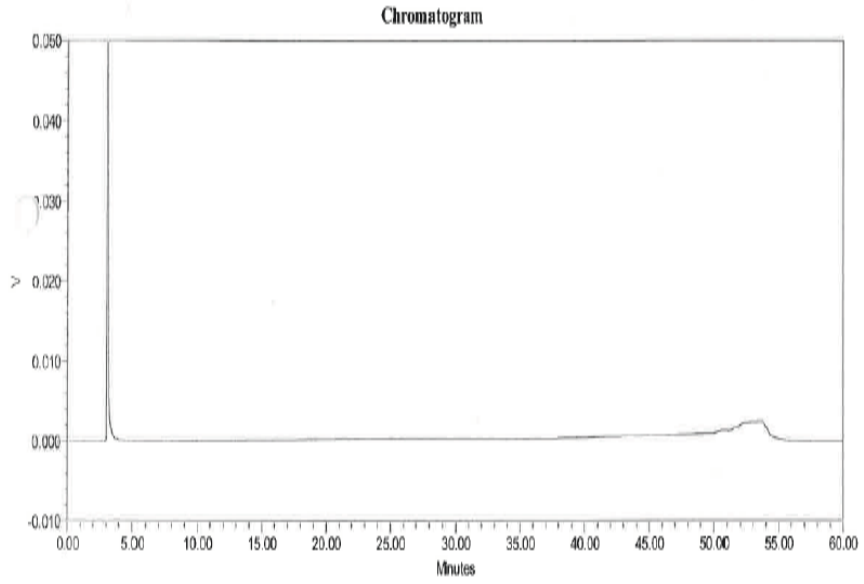
Conclusion

The system suitability complied with the acceptance criteria in all the validation parameters. All the validation parameters also complied with the acceptance criteria as mentioned in Table 1. Hence the analytical method for determination of related Substances of Pralatrexate in Pralatrexate Injection 20 mg/mL by High Performance Liquid Chromatography is suitable for its intended purpose.

14. Representative Chromatograms

Representative chromatograms of Blank (Diluent), System suitability solution, Standard solution, Placebo solution, Sample Solution, spiked sample solution and individual stock are presented as follows.

1. Chromatogram of Blank (Diluent)



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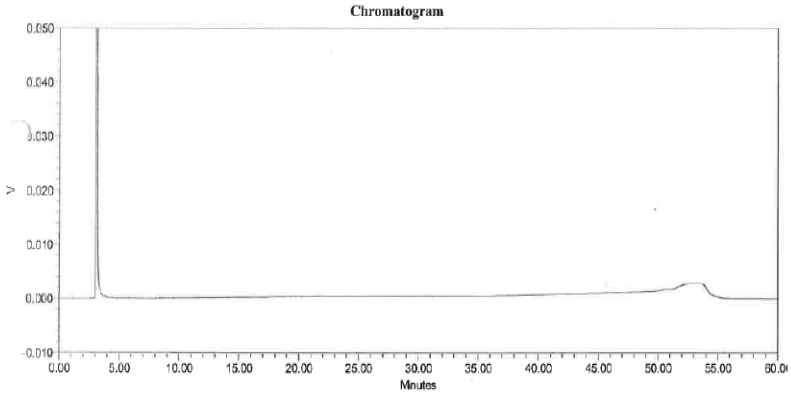
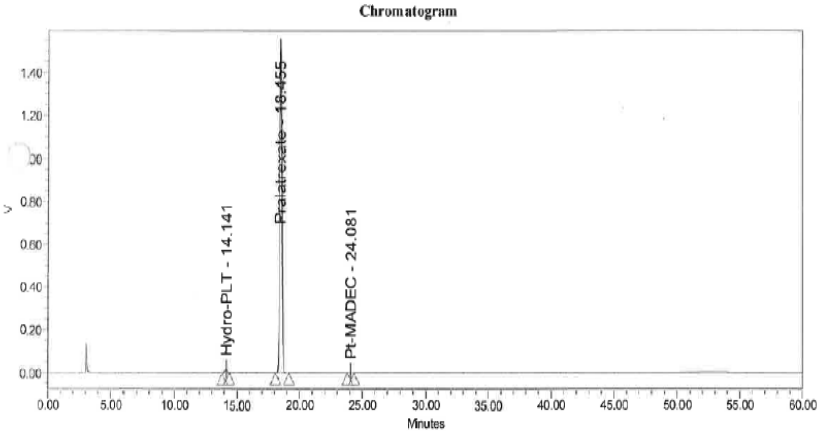
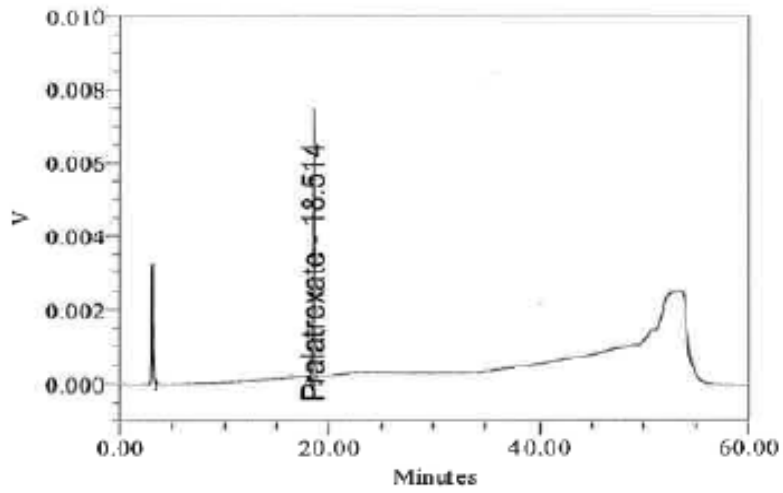
2. Chromatogram of Placebo Solution



3. Chromatogram of System Suitability Solution



4. Chromatogram of Standard Solution



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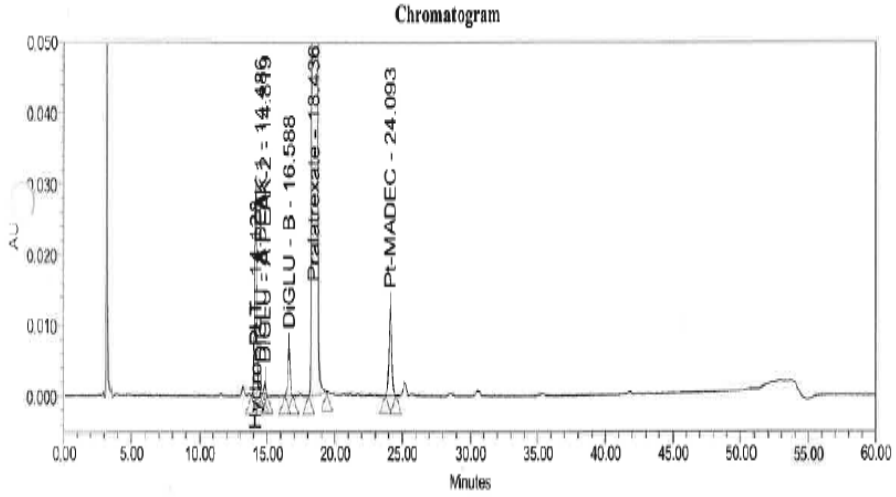
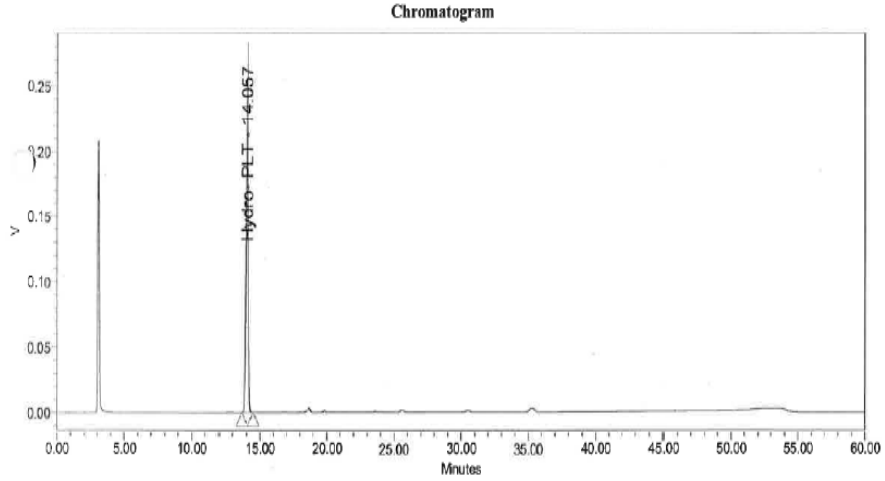
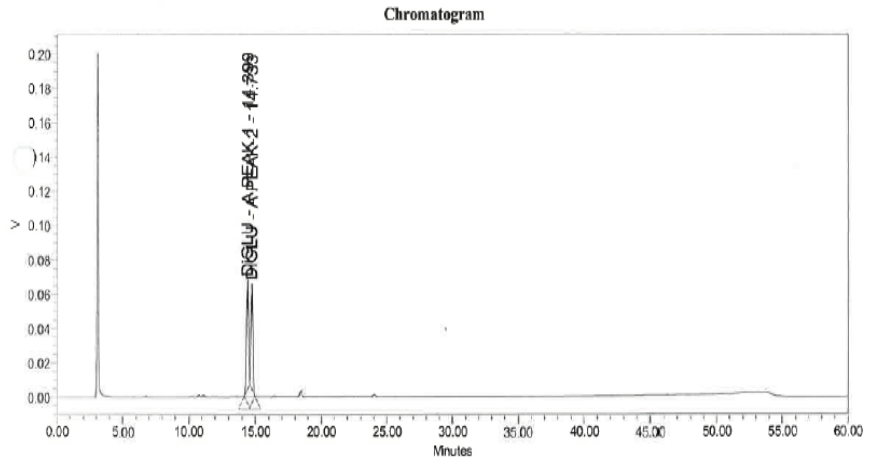
5. Chromatogram of spiked sample with all impurities



6. Chromatogram of Hydro-PLT Solutions



7. Chromatogram of DiGLU-A Solution



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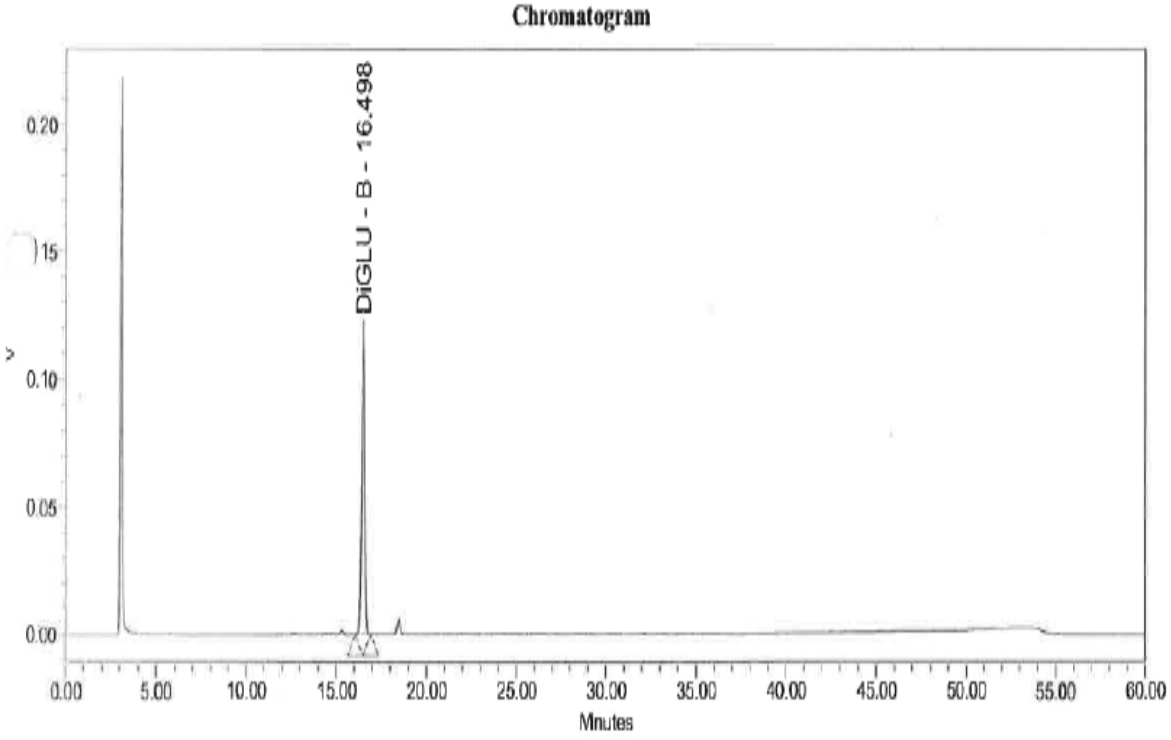
8. Chromatogram of DiGLU-B Solutions



9. Chromatogram of Pt-MADEC Solutions