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DETERMINATION OF ETRAVIRINE IN HUMAN PLASMA BY HPLC

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Abstract:
A simple bioanalytical HPLC method for the estimation of Etravirine in human plasma has been developed and validated. Fenofibrate was used as an Internal Standard . Extracted sample was eluted using C18 (250 x 4.6 mm, 5 µm) column. The mobile phase consisted of Acetonitrile:Water in the ratio of 85:15 v/v which was sonicated to degas and delivered at a flow rate of 1 ml/min at ambient temperature. The retention time of Etravirine and Fenofibrate was 5.32(± 0.1) and 3.58(± 0.1) minutes respectively. Studies were performed using an HPLC system equipped with a UV detector; the response was monitored at 308 nm. The method was validated according to MHLW (Japan) guidelines (2013). The data of linear regression analysis indicated a good linear relationship over the range of 0.16-0.64 µg/ml concentrations with correlation coefficient value of 0.993. The proposed method can be applied to the analysis of Etravirine from human plasma.

Key Words: *Etravirine, Human Plasma, HPLC, Bioanalytical study, Validation.*

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INTRODUCTION:

Etravirine [1-3], chemically known as 4-[6-Amino-5-bromo-2-[(4-cyanophenyl)amino] pyrimidin-4-yl]oxy-3,5-dimethylbenzonitrile, is a second-generation non-nucleoside reverse transcriptase inhibitor. Etravirine (ETRA) works by blocking the growth of HIV through binding directly to reverse transcriptase and obstructs the RNA-dependent and DNA-dependent DNA polymerase activities. It is soluble in Acetonitrile (ACN) and Mol.Weight is 435.28 g/mol. Pharmacokinetic data of Etravirine includes, Bioavailability : 90% , Protein binding : 99.9% , Cmax 0.41 µg/ml, Metabolism : Hepatic system by enzymes CYP3A4, CYP2C9 and CYP2C19, Half-life : 41 hours and Excretion : Fecal [1-2]

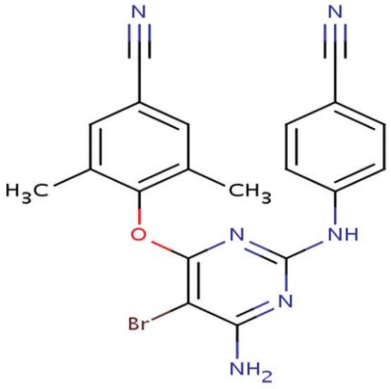

Fig.1 Structure of Etravirine

Etravirine is not official in IP/BP/USP. Literature survey reveals few analytical methods were reported for the determination of Etravirine by simple HPLC, stability indicating HPLC method [3-6], UPLC[7-8] , HPTLC[9], Quantification of Etravirine in Rat Plasma by LC-MS/MS[10]

MATERIALS AND METHODS:

Instrumentation

Bioanalytical assay of Etravirine was performed on HPLC(Make-JASCO) equipped with HiQSiL C18 column (250×4.6 mm;5µm particle size), Rheodyne injector and Jasco UV 2075 plus detector. The data acquisition was performed by Borwin chromatography software (version 1.5). Digital Balance Shimadzu make was used for weighing chemicals. Ultra-sonic bath sonicator was used for degassing of the mobile phase.

Materials

Etravirine used as working standard, was received as gift from HETERO Pharma, Hydrabad, India. Fenofibrate used as a Internal standard was received as gift from Lupin Ltd, Aurangabad ,India. Water (HPLC grade) generated by double distillation through ELGA system, All chemicals and reagents i.e. ACN (HPLC grade) and Chloroform were purchased from Loba Chemie.

Selection of Internal Standard (IS) –

Internal standard is a compound added to samples to improve the accuracy and precision of quantitation as well as the robustness of bioanalytical method. Darunavir, Nevirapine,Desloratidine,Telmisartan,Nebivolol HCl, Lurasidone,and Fenofibrate were tried as internal standard, among all Fenofibrate was selected as internal standard as peaks of the drug and IS were well resolved. Also, Fenofibrate was well extracted from extraction technique that is developed for extraction of Etravirine from human plasma.

Selection of Wavelength

From Standard stock solution further dilutions were done using acetonitrile and it was scanned over the range 200-400 nm.

Mobile Phase Preparation

85 ml of HPLC grade ACN was added to 15mL of HPLC grade water (85:15v/v ratio). The solution was further filtered through 0.45µm membrane filter and sonicated in sonicator bath for 10 min.

Preparation of stock solution –

Stock solution of ETRA and FENO (I.S) were prepared separately by transferring accurately weighed 10 mg of drug into a 10ml volumetric flask and making up volume with mobile phase to get concentration of 1000 µg/ml. Working stock solution for Etravirine was prepared by diluting appropriately stock solution to get final concentration 0.32-1.28 µg/ml and for FENO to get final concentration 500 µg/ml

Preparation of Spiked plasma sample

The reported plasma peak concentration values for Etravirine is 0.41µg/ml. On this basis, the linearity range was chosen as 0.16-0.64µg/ml. Spiked plasma was prepared by spiking 4.6 ml plasma with 0.2ml from each stock solution (4, 6 ,8, 10, 12, 14, 16 µg/ml) and 0.2ml of fenofibrate (500 µg/ml) as a internal standard separately .The content were mixed by Vortex mixer for 10 min. 1 ml of this solution was pipetted into separate test tube to which 3ml Chloroform was added. These solutions were gently mixed by Vortex Mixer and then were centrifuged for 15 min. Chloroform extraction step was performed twice. The clear chloroform layer was separated and allowded to evaporate on water bath then cooled at RT and further 0.5ml of mobile phase was added ,mixed properly and then injected. A blank plasma sample was treated similarly.

Method Validation

The method was validated in accordance with MHLW guidelines, Japan[11]. According to this guidelines there are parameters for bioanalytical method validation are

Selectivity:

The selectivity of the method was evaluated by analysing pooled plasma samples spiked at LLOQ.

Calibration Curve:
Linearity was tested for the range concentration 0.3-1.28ug/mL. Each sample in 5 replicates was analysed and peak area were recorded.

Accuracy and Precision:
Accuracy was measured by using minimum 5 determination per 4 concentration i.e. at LLOQ, LQC, MQC, HQC. The precision of this method was evaluated by % CV at different concentration levels.

Recovery:
It was evaluated by replicate analysis of at least 3 times each at 3 concentration levels (low-, mid-, and high-levels).

Carry over:
It was evaluated by analysing a blank sample following the highest concentration calibration standard.

Dilution Integrity:
It was evaluated by taking 1 mL spiked plasma of conc.1.12µg/mL and it was tested upon subsequent dilution.

Stability Study
The stability of the ETRA solutions and plasma samples was also evaluated during method validation. ETRA stability was evaluated using two concentration levels i.e. at LQC, HQC.
Three Types of stability studies were performed i.e. Freeze and Thaw stability, Short term stability and Long term stability.

- Short term stability - A stock solution was kept at room temperature for 4 hours and checked for its stability.
- Long term stability-A stock solution was kept in deep freezer for 14 days and checked for its stability.
- Freeze thaw stability-The stability of low and high quality concentration samples was determined after three freeze thaw cycles.

RESULTS AND DISCUSSION:

It was observed that ETRA showed considerable absorbance at 308nm. Hence this wavelength was chosen for detection.

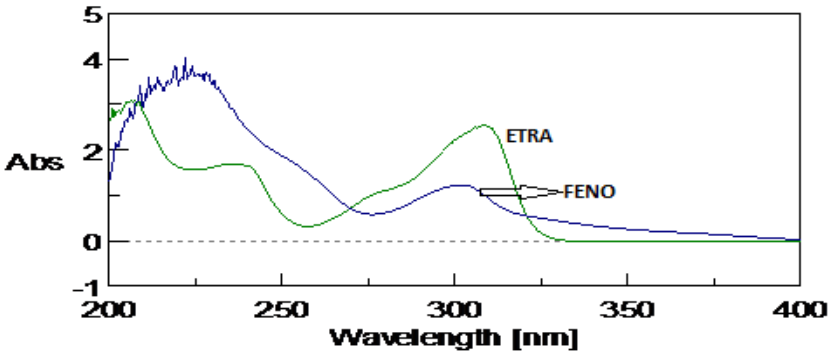

Fig.2: Overlay UV Spectra of ETRA (10µg/mL) and FENO (10µg/mL)

Fig 3: Chromatogram of blank human plasma.

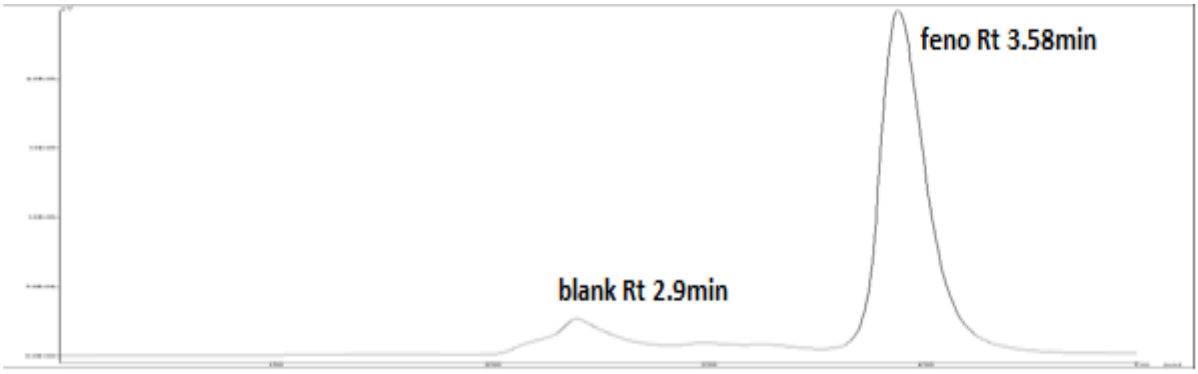

Fig 4: Chromatogram of zero human plasma

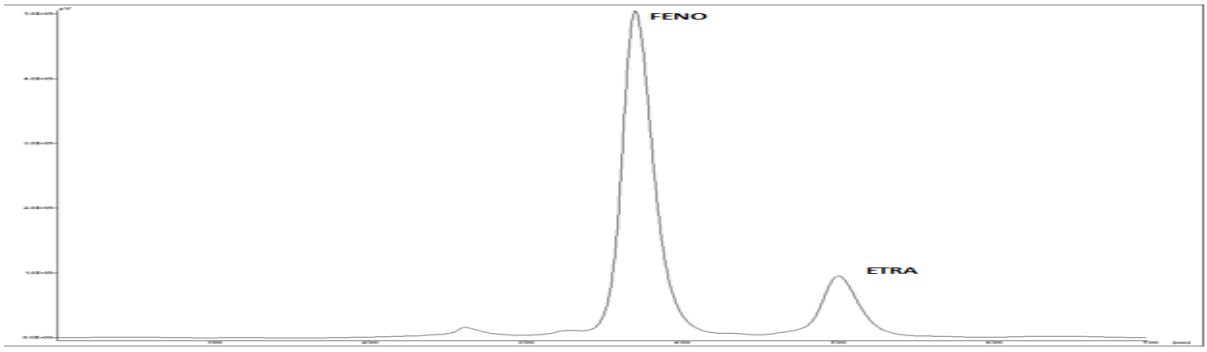

Fig. 5: Typical Chromatogram of blank human plasma spiked with Etravirine (Rt 5.32min) and Fenofibrate (I.S) (Rt – 3.58 min)

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability.

Selectivity

It was evaluated using blank plasma samples. The absence of interference at ETRA retention time was confirmed as shown in Table 1.

Table 1: Results for Selectivity of ETRA

Replicate No.	Nominal Conc. (LLOQ) (0.27µg/ml)				
	Peak area of ETRA	Peak area of FENO	Response Factor	Calculated Concentration	
				µg/ml	% Accuracy
1	78232.3	594032.3	0.131	0.314	116.38
2	80332.3	617985.3	0.149	0.310	115.13
3	74536.3	562365.3	0.132	0.315	115.00
4	87032.3	651395.3	0.143	0.318	117.79
5	73132.3	552956.3	0.132	0.315	116.80
6	78232.3	594032.3	0.121	0.314	115.38
Mean				**0.316**	**115.29**
SD				**0.044**	**3.63**
%CV				**3.17**	
Acceptance Criteria: The % CV should be within 15% of the analyte response at LLOQ. The % CV for LQC, MQC and HQC samples should be within 15.00 %.					

Linearity:
Linearity was tested for the range of concentrations 0.27-1.28 µg/ml. Each sample in five replicates was analyzed and peak areas were recorded. The response factor for each concentration was calculated by taking the peak area ratio of ETRA and FENO (I.S).

Table 2: Linearity Studies of ETRA spiked plasma

Replicates	Concentrations (µg/ml)							
	0.27	**0.32**	**0.48**	**0.64**	**0.82**	**0.96**	**1.12**	**1.28**
	Response Factor							
1	0.152	0.194	0.278	0.350	0.428	0.614	0.614	0.649
2	0.149	0.210	0.272	0.345	0.410	0.613	0.613	0.638
3	0.164	0.205	0.276	0.366	0.416	0.565	0.565	0.690
4	0.156	0.192	0.246	0.351	0.423	0.592	0.592	0.654
5	0.145	0.192	0.261	0.347	0.413	0.549	0.549	0.645
Mean	**0.153**	**0.198**	**0.263**	**0.348**	**0.452**	**0.538**	**0.591**	**0.657**
SD	**0.007**	**0.006**	**0.019**	**0.021**	**0.058**	**0.072**	**0.027**	**0.016**
%RSD	**4.73**	**3.26**	**7.25**	**6.25**	**13.00**	**13.55**	**4.65**	**2.49**

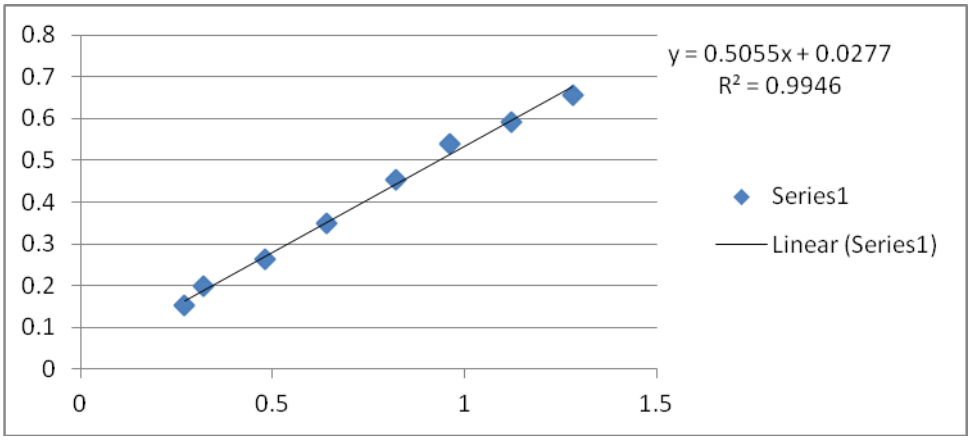

Fig.6: Calibration curve of spiked plasma Etravirine

Table 3: Accuracy Studies of ETRA

Replicates	Calculated conc			
	At LLOQ 0.29 µg/ml	**At LQC 0.59 µg/ml**	**At MQC 0.82 µg/ml**	**At HQC 1.12 µg/ml**
1	0.314	0.558	0.846	1.069
2	0.310	0.562	0.772	1.196
3	0.315	0.557	0.753	1.201
4	0.311	0.559	0.842	1.084
5	0.315	0.557	1.08	1.146
Mean	**0.312**	**0.558**	**0.834**	**1.119**
SD	**0.04**	**0.04**	**0.108**	**0.055**
%CV	**12.82**	**7.27**	**12.95**	**4.23**
%Mean Accuracy	**114.81%**	**93.31**	**101.28**	**99.91**
Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15.00 %.				

Accuracy:
Accuracy was measured by using minimum 5 determination per 3 concentration i.e. at LQC, MQC, HQC level ranged from 93.31%-101.28%, which is within acceptance limit 85%-115% while at LLOQ level it was found to be 114.81% which is within acceptance limit 80%-120%, as shown in Table 3.

Precision:
A) Inter day Precision
The Inter day Precision was evaluated in five replicates for four different concentration of ETRA on three consecutive days (fresh samples were prepared every day.) The % CV of calculated concentration for all Quality control samples of LLOQ, LQC, MQC, HQC concentration level ranged from 3.36-12.01% as shown in Table 4.

B) Intraday precision
Repeatability of the method was evaluated in five replicates on the same day for four different concentration of ETRA (0.27-1.12µg/mL). The % CV of calculated concentrations for all quality control samples at LQC, MQC, HQC concentration levels ranged from 6.34-10.21% , which is within acceptance limit 15%, and at LLOQ levels it is found to be 14.01% as shown in Table 5

Recovery
The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against un-extracted quality control samples at HQC, MQC and LQC levels. Recovery from human plasma samples was evaluated in triplicate for each three concentrations of ETRA (0.59,0.82 and 1.12ug/ml).

Table 4: Results of Interday Precision for ETRA

Conc Level	% CV		
	Day 1	Day 2	Day 3
At LLOQ	11.59	8.95	5.31
At LQC	8.16	4.30	5.81
At MQC	3.82	5.97	3.36
At HQC	6.39	7.94	7.61
Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15.00 %.			

Table 5: Results of Intraday Precision for ETRA

Con.Level	At LLOQ	At LQC	At MQC	At HQC
%CV	14.01	10.21	6.34	9.22
Acceptance Criteria: The % CV for LQC, MQC and HQC samples should be within 15.00 %.				

Table 6: Results of Recovery for ETRA

Conc level	Area of ETRA		Area of FENO (I.S)	
	Standard	Spiked plasma	Standard	Spiked plasma
LQC	182869.1	145456.3	532446.3	499204.1
MQC	280271.3	221015.9	542469.7	506236.3
HQC	401226.6	304270.7	532600.3	509113.1
Overall % mean Recovery	78.07		94.22	

Table 7: Results of Carry-over for ETRA

Replicates	Area of ETRA		Area of FENO (I.S)		% Carryover	
	At LLOQ	At Blank	At LLOQ	At Blank	ETRA	FENO
1	88632.3	10326.3	533689.3	29563.3		
2	84892.3	10115.3	556895.1	27583.0		
3	80232.3	9632.80	552353.2	28300.3	**11.82%**	**5.20%**
Mean	**84585.6**	**10024.4**	**547645.8**	**28482.2**		
SD	**4208.3**	**722.8**	**25676.2**	**1002.6**		
%RSD	**4.97**	**7.03**	**4.64**	**3.57**		
Acceptance Criteria: The % carryover should not be greater than 20% of the analyte response at LLOQ						

Table 8: Results of Dilution Integrity for Etravirine

Conc µg/ml	Calculated conc	% Recovery
0.37	0.36	98.64
	0.36	97.29
	0.32	87.29
	0.38	102.73
	0.35	94.56
MEAN	**0.35**	96.08
%CV	**5.96**	
Acceptance Criteria: The % CV should be within 15.00 %		

Carry-over:
Carry-over evaluated by analysing a blank sample following the highest concentration calibration standard. % Carryover for ETRA and FENO (I.S) is 11.82% and 5.20% resp ,which is within limit as shown in Table 7

Dilution integrity
Dilution integrity were evaluated by taking 1 mL spike plasma of conc. 1.12 µg/mL and it was diluted by adding 2 mL plasma in it. % Recovery of diluted samples was noted as shown in Table 8

Stability
Drug Stability in biological fluid is a function of storage conditions, chemical properties of drug, the matrix and the container system. Stability procedure should evaluate the stability of analyte during sample collection and handling after long term (frozen at intended storage temp.) and short term (room temp.) storage conditions. The results are summarized in the Table 9

A. Freeze and thaw stability-
Freeze and thaw stability of spiked quality control samples was determined after 3 Freeze and thaw cycles stored at -5^{0}C ±0 ^{0}C. Compared them to the freshly spiked quality control sample to assess stability. The mean % stability for HQC(1.12 µg/mL) and LQC(0.59 µg/mL) was found to be 96.91 % and 99.82% respectively

B. Long Term stability-
Long-term stability of the LQC and HQC was determined for a period of 14 days stored at 4^0 C, comparing them against the freshly prepared stock solution assessed for stability. The % mean stability for HQC (1.12µg/ml) and LQC (0.59µg/ml) are found to be 99.10 % and 96.61 % respectively.

C. Short term stability-
Short term temp.stability of spiked quality control samples was determined for a period of 4 hrs. stored at room temperature. Comparing them against the freshly spiked quality control samples assessed stability. The % mean stability for HQC and LQC are found to be 95.49%, 100.36% respectively.

D Stock solution stability:
Stock solution stability of the drug and IS was determined for 2 hrs at room temperature. Comparing them against the freshly weighed stock solution assessed for stability. The % mean stability for ETRA at HQC & LQC levels was found to be 97.32 % and 93.22% respectively.

Table 9: Summary of stability studies for Etravirine and IS

Stability	Conc (µg/mL))	Mean Stability (%)	% R.S.D.
Freeze thaw stability (three cycles)	**0.59**	99.82	3.47
	1.12	96.91	4.92
Short term stability (for 4h at RT)	**0.59**	100.36	10.71
	1.12	95.49	9.06
Long term stability (for 14 days at 4^0 C)	**0.59**	96.61	3.54
	1.12	99.10	6.03
Stock solution stability (for 2h)	**0.59**	93.22	13.90
	1.12	97.32	4.18
Acceptance Criteria	**85-115%**		**≤ 15%**

DISCUSSION

There is research article available in literature for Quantification of Etravirine in Rat Plasma by LC-MS/MS and application to a Pharmacokinetic Study[10] . So we have decided to work on human plasma. In current work we have used simply ACN:Water (85:15v/v) as mobile phase and UV detector .Thus the developed method is rapid and simple.

CONCLUSION:

A new, simple and rapid method for the quantification of ETRA in human plasma using HPLC with UV detection has been developed. The method reported here uses a simple and effective extraction technique with good and reproducible recovery. It is suitable for application to a pharmacokinetic, bioequivalence studies for the estimation of ETRA from plasma.

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