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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF ETRAVIRINE IN BULK AND TABLET DOSAGEFORM

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ABSTRACT

A simple high-performance liquid chromatographic (HPTLC) method has been developedand validated for simultaneous determination ofEtravirine in bulk andtablet dosage form. The method employed pre-treated HPTLC plates with silica gel with fluorescent indicator with layer thickness (0.2 mm) 10 cm x 10 cm aluminium (E-Merck-KgaA) as the stationary phase. The mobile phase used was a mixture of ethyl acetate: toluene (6:4 v/v). The detection of spot was carried out at 254 nm. Thecalibration curve of Etravirine was found to be linear between in the range of125 to 750ng/spot with regression coefficient0.9930.The Limit of detection (LOD) was found to be 8.36ng/spot while the Limit ofquantification (LOQ) was found to be 25.33ng/spot for Etravirine.The proposed method can be successfully used to determine the drug content ofmarketed formulation. The accuracy of the proposed method was determined by recoverystudies and found to be 98.20 to 101.29%. The proposed method is applicable to routineanalysis of Etravirine in bulk and pharmaceutical formulations. The proposed method wasvalidated according to various ICH parameters like linearity, accuracy, precision, specificity,LOD, LOQ, range and solution stability.

KEY WORDS

Etravirine, HPTLC, method development, ICH guidelines

1. INTRODUCTION

During the past decade HIV infection has become, at least in developed countries, a largely manageable but incurable disease. This is due to the advent of Highly Active Anti Retroviral Therapy (HAART), in which patients are treated with a cocktail of drugs designed to reduce their viral loads to extremely low levels [1]

Etravirine[2] is a second-generation non-nucleoside reverse transcriptase inhibitor(NNRTI),designed to be active against HIV with mutations that confer resistance to the two most commonly prescribed first-generation NNRTIs, mutation K103N for Efavirenz. This potency appears to be related to Etravirine's flexibility as a molecule. Etravirine is a diarylpyrimidine (DAPY), a type of organic molecule with some conformational isomerism that can bind the enzyme reverse transcriptase in multiple conformations, allowing for a more robust interaction between Etravirine and the enzyme, even in the presence of mutations.In turn, HIV's genetic material cannot be incorporated into the healthy genetic material of the cell, and prevents the cell from producing new virus [3].Etravirine is chemically4-[[6-amino-5-bromo-2-[(4-cyanophenyl) amino]-4-pyrimidinyl] oxy]-3, 5-dimethylbenzonitrile and its chemical structure depicted in **Figure 1**.

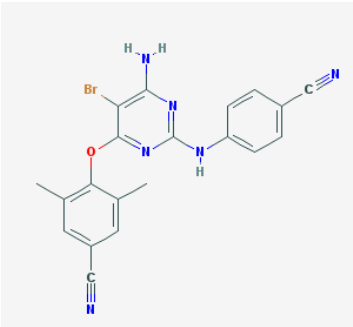

Figure 1: Structure of Etravirine

The literature survey reveals that Etravirine was analyzedby a few chromatographic methods for the quantification of Etravirine by LCMS, HPLC and UPLC[4-6]. However, HPTLC methodhad not been reported till date in literature for analysis ofEtravirine as bulk drug or in formulations.

HPTLC is a widely used analytical technique due to itsadvantages of low operating cost, high sample throughput,and need of minimum sample preparation. The majoradvantage of HPTLC is that several samples can be runsimultaneously using a small quantity of mobile phase unlikeHPLC, thus reducing the analysis time and cost per analysis [7].

The developed LC method is validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness as per ICH recommended conditions [8].

2. EXPERIMENTAL

2.1. Materials and Methods

Etravirine was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.Commercially available Tablets were purchased from local market. Toluene and methanol HPLC grade and Ethyl acetate AR grade were obtained from Rankem, RFCL Ltd., New Delhi. Water was prepared by using Millipore Milli Q Plus water purification system.

2.2 Preparation of mobile phase: The mobile phase was prepared by mixing 6.0 mL ethyl acetate with 4.0 mLtoluene. The mobile phase was transferred into a twin-trough chambercovered with lid and allowed to stand for 30 min before use.

2.3 Preparation of Standard stock solution of Etravirine: Etravirine (100mg) was accurately weighed and transferred into 100 mL volumetricflask,and dissolved in methanol. The volume was made up to the mark withmethanol. Aliquotwas further 4 times diluted with methanol to get the finalconcentration of 250µg/mL.Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0µL) were applied to the HPTLC plateas bands of 6 mm.

2.4 Etravirine Sample Solution Preparation: Twenty tablets, each containing 600 mg of Etravirine, were weighed and theiraverage weight was calculated. The tablets were finely powdered and powderequivalent to 100 mg Etravirine was accurately weighed and transferred in to 100mL volumetric flask. Methanol (60 mL) was added to it and shaken for 10minutes. The volume was made up to the mark with methanol. The solutionwas sonicated for 30min, filtered through the Whatman no.41 filter paper.

Aliquot was further 4 times diluted with methanol to get the final concentrationof 250µg/mL Etravirine. An aliquot (2 µL equivalent to500ng/spot Etravirine) wasapplied to the HPTLC plate.

2.5 Pre-treatment of HPTLC plates:

HPTLC plate was placed in twin-trough glass chamber containing methanolas mobile phase. Methanol was allowed to run up to the upper edge of plate(ascending method). The Plate was removed and allowed to dry in oven at60^{0}C for 5 min. For the actual experiment the plate was allowed toroom temperature and used immediately.

2.6 Instrumentation

The instrument used in the present studywas Camag HPTLC with Linomat V auto spotter and Camag Scanner-III,Cagmag Twin trough chamber ofappropriate size (20X 20), Analyticalweighing balance (Shimadzu AX 200),Sonicator (model SONICA 2200MH)were used throughout the experiment.CagmagWincats software was used foracquisition, evaluation and storage ofchromatographic data.HPTLC plates used were silica gel with fluorescent indicator 254 nm, layer thickness (0.2 mm) 10 cm x 10 cm aluminium (E-Merck-KgaA).

3. METHOD DEVELOPMENT

3.1 Selection of mobile phase

Resolution is the most important criteria for the method, it is imperative toachieve good resolution among the compounds. As per the value of pKa andsolubility of compound various composition of mobile phase were tried.The chromatographic conditions were optimized with mobile phase

consistingof ethyl acetate: toluene (6:4 v/v), which was found satisfactory to obtainsharp, well defined Etravirine peak with better reproducibility and repeatability.

3.2Chromatographic separation

The chromatographic separation was achieved on HPTLC plates using mobilephase ethyl acetate: toluene (6:4 v/v). Etravirine reference standard solution wasprepared using methanol as solvent. From the prepared standard solution,appropriate volume of aliquots were applied to silica gel 60 F254 HPTLCplates (10 cm x 10 cm) as spot bands of 6mm using LINOMAT V. Mobilephase components were mixed prior to use and the development chamberwas left for saturation with mobile phase vapors for 10 min before each run.Development of the plate was carried out by the ascending technique to amigration distance of 7 cm. Then the plates were allowed to dry. All theanalysis was carried out in a laboratory with temperature control (25 ± 2^0C).

Densitometry scanning was done in absorbance mode at 254 nm using adeuterium lamp. The slit dimensions were set at 6 mm x 0.30 mm, thescanning speed of 10 mm/s, and the data resolution at100µm/step. Singlewavelength detection was performed since the main components were onlyanalyzed.

3.3 Analysis of Tablet Dosage Form: The plate was developed and analyzed as above by applying previously prepared sample solution. The chromatogram was recorded. The peak areawas noted and amount of Etravirine was calculated from the regression equation.

The typical chromatogram obtained for Etravirine standard and samplefrom final HPTLC conditions are depicted in **Figure 2 and 3**respectively**.**

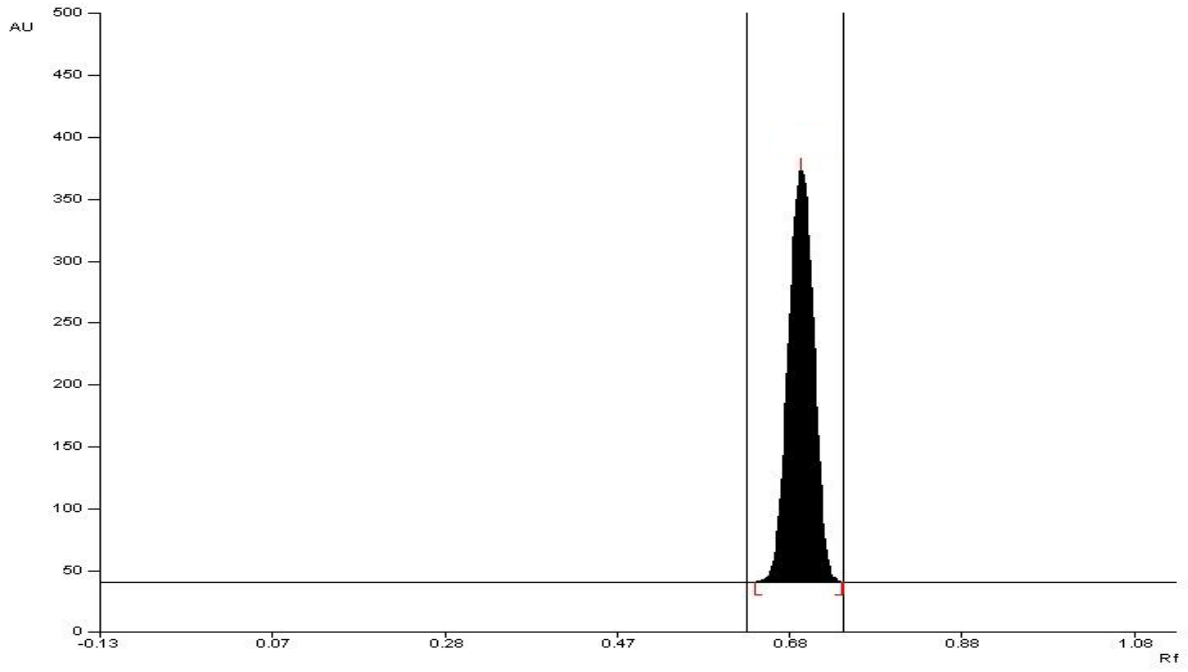

Figure 2: HPTLC Chromatogram of Etravirine (750 ng/spot) standard withcorresponding R_f at 254 nm

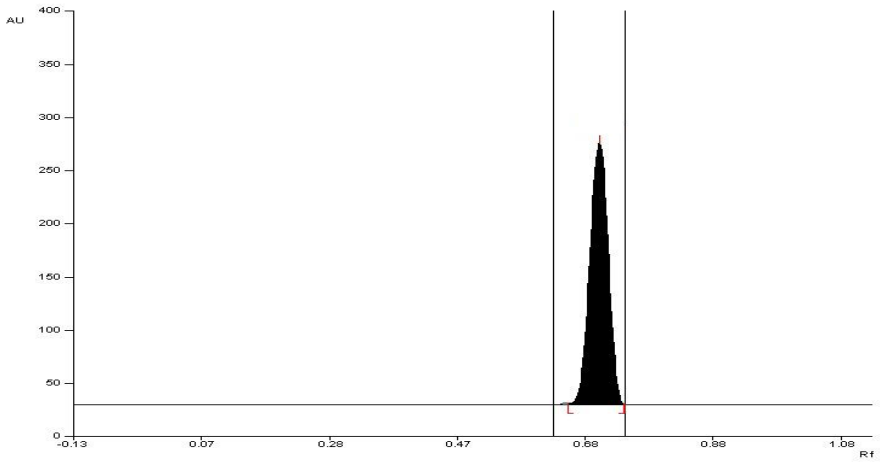

Figure 3: HPTLC Chromatogram of Etravirine (500 ng/spot) tablet samplewith correspondingR$_f$ at 254 nm

4. METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studiedwere solution stability, specificity, linearity, accuracy, precision, limit ofdetection, limit of quantitation and robustness.

4.1 Solution Stability

Sample solutions were kept at 25^{0}C (24 hours) and 2 - 8^{0}C (3 days),respectively. Assay of initial time period was compared with these two timeperiods. The falls in the assay values were evaluated. The difference betweenassays should not be more than 2 % for formulation, and 0.5% for API.

4.2 Specificity

Specificity of an analytical method is its ability to measure the analyteaccurately and specifically in the presence of component that may beexpected to be present in the sample matrix. Chromatograms of standard andsample solution of Etravirine were compared, and peak purity spectra at threedifferent levels i.e., peak start (S), peak apex (M) and peak end (E) of a spotwere recorded in order to provide an indication of specificity of the method.

4.3 Linearity (Calibration curve)

Standard stock solution containing 1000µg/mL Etravirine was prepared inmethanol and 4 times diluted. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0µL) wereapplied to the HPTLC plate to deliver 125, 250, 375, 500, 625 and 750ng of Etravirine per spot. The plate was developed and analyzed. The chromatograms were recorded and the peak areas were noted.

Calibration curve was constructed by plotting peak area versus concentration,and the regression equation was calculated. Each response was average ofthree determinations.

4.4 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recovery of Etravirine by the standard addition method. Known amount of standard solutions of Etravirine (equivalent to 0, 125, 250 and 375ng/spot) were applied to the sample spot of Etravirine (250ng/spot) on the plate. Each solution was applied in triplicate. Theplate was developed and analyzed. Thepercentage recovery was calculated by measuring the peak areas and fittingthese values into the regression equation of the calibration curve.

4.5 Precision

The repeatability of measurement of peak area was checked by repeatedly (n= 6) measuring area of one band of Etravirine (500ng/spot), while repeatability ofsample application was checked repeatedly (n = 6) by measuring area of sixbands having same concentration of Etravirine (500 ng/spot) applied on the sameplate without changing the position of plate. The intra-day and inter-dayprecisions of the proposed method was determined by measuring thecorresponding responses 3 times on the same day and on 3 different daysover a period of 1 week for 3 different concentrations of Etravirine (250, 500 and750ng/spot). The results were reported in terms of relative standarddeviation.

4.6 Limit of Detection and Limit of Quantification:

Limit of detection (LOD) and the limit of quantification (LOQ) were calculatedusing the standard deviation of response (σ) and slope (S) of the calibrationcurve.

LOD = 3.3 x σ/S

LOQ =10 x σ/S

4.7 Robustness

The robustness was studied by analysing the samples of Etravirine by deliberatevariation in the method parameters. The change in the response of Etravirine wasnoted. Robustness of the method was studied by changing the extraction timeof Etravirine from tablet dosage form by ± 2 min, composition of mobile phase by ±2% of organic solvent, development distance by ± 1 cm, wavelength by ± 2nm and temperature by ± 2^0C. The changesin the response of Etravirine werenoted and compared with the original one.

5. RESULTS AND DISCUSSION:

5.1 Solution Stability

The change in assay results after storage at 25°C (24 hours) and 2-8°C (3days) was evaluated. It was found that the difference in assay results was notmore than 2 % for formulation, and 0.5% for API, indicating stability of Etravirine solution.

5.2 Specificity

The proposed method was found to be specific as no interference ofexcipients or impurities was found in separation and determination of the peakpurity of Etravirine, as r(S, M) = 0.9999 and r(M, E) = 0.9998, andgood correlation (r = 0.9999 and 0.9998) was obtained between standard andsample spectra of Etravirine, respectively. The peak purity and correlation > 0.99indicated that the method is specific. The peak purity of the spectra Etravirine from tablet dosage form is shown in the **Figure 4.**

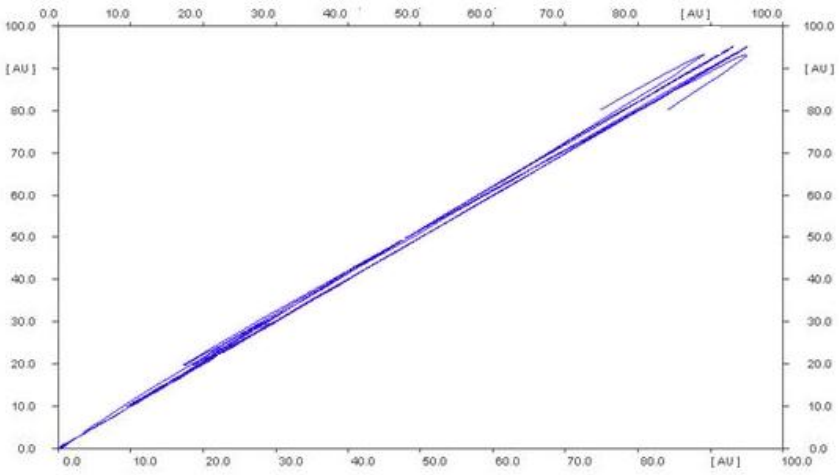

Figure 4: Peak purity spectra of Etravirine from tablet dosage form

5.3 Linearity

Linear correlation was obtained between peak area and concentration of Etravirine in the range of 125-750ng/spot. The linearity of the calibration curve wasvalidated by the value of correlation coefficients of the regression (r). Theoptical and regression characteristics are listed in **Table 2.**The 3D Chromatogram showing peaks of Etravirine standards in different concentrations are illustrated in **Figure 5**and the Calibration curve is shown in **Figure 6.**

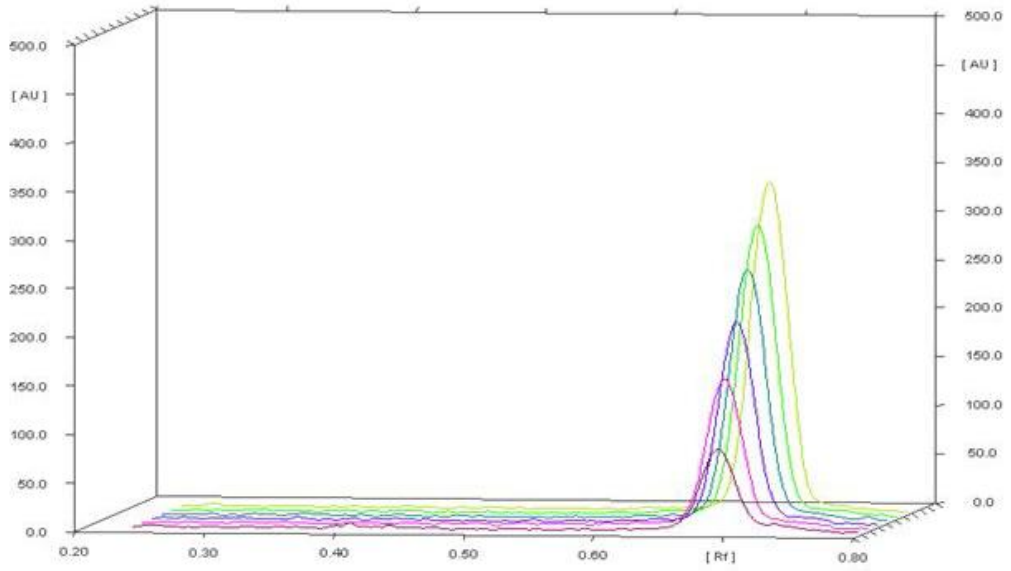

Figure 5: 3D Chromatogram showing peaks of Etravirine standards in different Concentrations

Table 2: Optical and regression characteristics (n=3)

Parameter	ETRAVIRINE
Linearity range (ng/spot)	125-750
Linearity equation	y = 9.024x + 734.4
LOD (ng/spot)	8.36
LOQ (ng/spot)	25.33
Correlation coefficient (r)	0.9930

5.4 Accuracy (% Recovery)

Accuracy study was carried out by the standard addition method. The percentrecovery was found in the range of 98.20 – 101.29 % for Etravirine, which indicatedaccuracy of the method. The results are tabulated in **Table 3.**

Table 3: Results of recovery study (n=3)

Drug	Amount taken (ng/spot)	Amount added (ng/spot)	Amount found (ng/spot)	Recovery ±SD,%	%RSD
	250	0	245.52	98.20±1.43	1.43
ETRAVIRINE	250	125	379.86	101.29±0.69	0.69
	250	250	496.64	99.32±0.86	0.86
	250	375	616.92	98.70±1.64	1.64

5.5 Precision (Repeatability)

The % RSD of the repeatability of measurement of peak area was found to be0.77; while of the repeatability of sample application was found to be 1.80 for Etravirine. The % RSD for intra-day precision was found to be in the range of 0.65 -0.92 %; while inter-day precision was found to be in the range of 0.90 - 1.24% for Etravirine, which indicated that the method was precise.The results are tabulated in **Table4** and results for intra-day and inter-day precision is summarised in **Table 5.**

Table 4: Results of repeatability (n=6)

Drug	ETRAVIRINE	
	Measurement of peak area	Sample application
1	4275	4321.2
2	4234.6	4286.7
3	4284.2	4354.3
4	4221.3	4408.4
5	4312.3	4186.4
6	4264.6	4257.9
Mean	4265.43	4302.48
SD	33.12	77.41
% RSD	0.77	1.80

Table 5: Results of Intra-day and Inter-day precision (n=3)

Drug	Concentration (ng/spot)	Intra-day precision		Inter-day precision	
		Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
ETRAVIRINE	250	3077.0 ± 27.38	0.89	3122.4 ± 28.41	0.91
	500	5418.0 ± 35.21	0.65	5346.6 ± 48.11	0.90
	750	7310.2 ± 67.56	0.92	7245.4 ± 89.96	1.24

5.6 Limit of detection and limit of quantification

The Limit of detection (LOD) was found to be 8.36ng/spot while the Limit ofquantification (LOQ) was found to be 25.33ng/spot for Etravirine.

5.7 Robustness

The method was found to be robust as the results were not significantlyaffected by slight variation in extraction time, composition of mobile phase,development distance, wavelength and temperature.

5.8 Analysis of Tablet Dosage Form

The proposed HPTLC method was successfully applied for determination of Etravirine from tablet dosage form. The percentage of Etravirine was found to besatisfactory, which was comparable with the corresponding label claim. The results are tabulated in **Table 6.**

Table 6: Analysis results of tablet dosage form (n=3)

Drug	Labelled amount (mg)	Amount found (mg)	Assay %± SD
ETRAVIRINE	600	594.66	99.11±1.70

6. CONCLUSION:

A high performance thin layer chromatographic method has been developedand validated for the determination of Etravirine from tablet dosage form. Themethod was found to be specific as there was no interference of excipientsand impurity. The proposed method was found to be simple, accurate,precise, sensitive and robust. Hence, it can be used successfully for theroutine analysis of Etravirine in pharmaceutical dosage forms.

7. REFERENCES

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