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HPTLC method Development, Validation for Simultaneous Determination of Efavirenz, Emtricitabine and Tenofovir in combined tablet formulation and Forced Degradation Studies

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ABSTRACT

A simple, sensitive, accurate and precise high performance thin layer chromatographic method was developed and validated for simultaneous determination of efavirenz (EFA), emtricitabine (EMT) and tenofovir (TEN) in combined tablet formulation and forced degradation studies were performed as per ICH guidelines. Precoated silica gel 60F 254 was used as stationary phase and the mobile phase used was chloroform: methanol (90:10), gives high resolution for each drug. The densitometric evaluation of each drug was carried out at 262 nm. The developed method was simple, accurate and is suitable for analysis of the drugs and degradation products in stability studies of samples.

Keywords: HPTLC; Forced degradation studies; efavirenz (EFA); emtricitabine (EMT); tenofovir (TEN); ICH guidelines.

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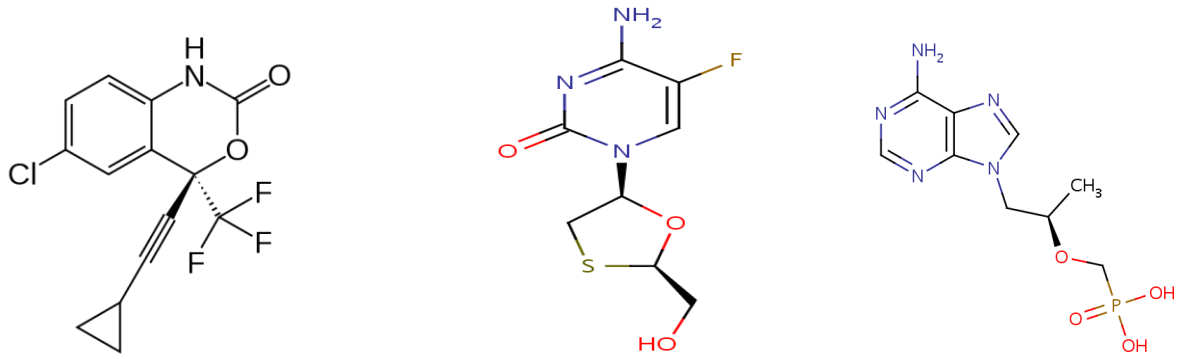
INTRODUCTION

Tenofovir is chemically known as (S)-6-chloro-4-(cyclopropylethynyl)-1, 4-dihydro-4-(trifluoromethyl)-2H-3, 1benzoxazin-2-one. Efavirenz is chemically known as [[(1R)-2(6-Amino-9H-purin-9-yl)-1-methylethoxy] methyl] phosphonate, bis (isopropyl oxy carbonyloxy methyl ester), fumarate. Emtricitabine (–)-4-Amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3oxathiolan-5-yl]pyrimidin-2(1H)-one;(–)-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3oxathiolan- 5-yl]cytosine; (-)-2',3'-dideoxy-5-fluoro-3'thiacytidine. The structures of these drugs are depicted in Figure 1.The fixed dose anti-retroviral combination of efavirenz, tenofovir and emtricitabine was approved by FDA for the treatment of HIV. Review of literature reveals that analytical methods such as RP-HPLC [1,] HPTLC [2], HPLC in plasma[3] and HPLC after solid phase extraction and LC-MS-MS [4] were reported for emtricitabine and tenofovir in combination. Methods like HPLC with post column photochemical derivatization[5], Isocratic mode HPLC-DAD detection method[6], LC[7,] stability-indicating LC-PDA-MS[8] and HPTLC[9] were reported for efavirenz. Many methods such as LC in human plasma[10], MALDI-TOF [11] and RP-HPLC method was reported for efavirenz alone[12] and in combination with other agents. Thus, here is an attempt to develop a new, simple, less time consuming and less expensive specific method of analysis for the given ternary combination of drugs by using HPTLC method and to perform its forced degradation studies as per the ICH guidelines [13-17].



(a) **(b)** **(c)**

Figure 1: Structure of (a) Efavirenz, (b) Emtricitabine (c) Tenofovir

MATERIALS AND METHODS

Tenofovir and emtricitabine were obtained from Cipla Ltd, Mumbai and efavirenz from Aurobindo Pharma Ltd. A.P., as free gift samples, Methanol (GR), chloroform (GR), hydrogen peroxide, hydrochloric acid and sodium hydroxide of Merck and Qualigens Fine Chemicals were used. Double distilled water and Whatmann filter paper No. 41 was used.Marketed formulation

containing EFA (600mg), TEN (300mg) and EMT (200mg) with a brand name Viraday manufactured by Cipla Ltd, Goa was used for the analysis.

Instrumentation

To carry out HPTLC analysis silica gel 60 F_{254} TLC plates 20 cm × 10 cm aluminum backed plates coated with 0.2 mm layer thickness of Merck, Darmstadt, Germany was used. Camag LINOMAT V sample applicator, CATs III scanner, and twin trough chamber was used. Hamilton syringe (100µl) and Camag WINCATS software version 4 was used for the study. Ultra-sonicator of Toshcon was used for extraction of drugs from tablet.

Chromatographic conditions

The chromatographic conditions maintained were Merck precoated silica gel 60F_{254} aluminum sheet (20×10 cm) was used as stationary phase, plate activation was done at 50 ^{0} C for 5 minute in hot air oven. Mobile phase consists of chloroform: methanol (9 mL: 1 mL v/v). Chamber saturation time was 30 minutes, number of tracks were 18, track distance from the plate edges was 15 mm, application height was 10 mm, band width was 8 mm, distance between bands was 4.5 mm. Densitometric scanning was performed using a Camag TLC scanner III in the absorbance mode at 262 nm for all measurements having slit dimension of 6×0.45 mm and operated by the WINCAT'S software version 4. The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm.

Preparation of standard

Standard stock solutions of EMT, TEN and EFA were prepared separately in methanol by dissolving 10 mg in 10 ml volumetric flasks to obtain 1000µg/ml concentration.

Then 1 ml of each solution was diluted to 10 ml with methanol to give 100 µg/ml concentrations. The standard solutions were applied to Merck precoated silica gel 60F_{254} aluminum sheet (20×10 cm) in the form of 8 mm bands to apply 132-726 ng/band for EFA, 132-462 ng/band for EMT and 132-462 ng/band for TEN, respectively. The plate was developed on mobile phase containing chloroform: methanol (9 mL: 1 mL v/v)) after development the well resolved bands of drugs were scanned at 262 nm, which shows linearity in the range 132-726ng for EFA, 132-462 ng for EMT and 132-462 ng for TEN with regression coefficient 0.998, 0.997 and 0.996 for EFA, EMT and TEN respectively.

Preparation of sample

Twenty tablets were weighed and finely powdered. Amount of tablet powder equivalent to 60mg of efavirenz, 20mg of emtricitabine and 30mg of tenofovir were weighed accurately, transferred to 100 ml volumetric flask and shaken with 50ml methanol for 15 min. Volume was made up to

100 mL with methanol and ultrasonicated for 15 min. Solution was then filtered through Whatmann filter paper No. 41. The solution was suitably diluted with methanol to get concentration 60ng/µl of EFA, 20 ng/µl of EMT and 30ng/µl of TEN. 10 µl of each drug was applied in the form of bands on the TLC plate in order to get 600 ng, 200ng and 300ng /spot of EFA, EMT and TEN, respectively. The plate was developed using mobile phase, containing chloroform: methanol (9 mL: 1 mL v/v)).

Estimation of each drug in tablets by proposed method

The sample solution of tablet containing 600 ng, 200ng and 300ng of EFA, EMT and TEN, respectively, were applied on the TLC plate in the form of 8 mm wide bands. After development the bands were scanned at 262 nm. The results of assay were in the range of 98.40% to 101.93% with 1.199, 1.144 and 1.115 as the values of standard deviation for EFA, EMT and TEN, respectively.

Forced degradation studies

The drugs were subjected to stress conditions of acid hydrolysis, alkali hydrolysis, oxidation, thermal degradation and neutral degradation Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and intrinsic stability of the molecule. Specificity is the ability of the method to measure the analyte response in presence of its potential impurities.

All stress studies were performed to a mixture containing 100 ng, 150 ng and 300 ng/µl of EMT,TEN and EFA, respectively. Acid and alkali hydrolysis were carried out in 1N HCl and 1N NaOH, separately. The mixture was kept at 100^0C for 1 hr on water bath. For oxidative degradation, 3 ml of 3% H_2O_2 added and mixture was kept at 100 ^{0}C for 1 hr on water bath. For neutral stress study, solution of mixture was prepared in distilled water and kept at 100ºC for 1 hr. For thermal degradation tablet powder was left at 100^0 C for 24 hrs. The sample was then treated to obtain solution containing 600µg, 150µg and 100µg/ml of EFA, TEN and EMT, respectively.

METHOD VALIDATION

Linearity and range:

Linearity was determined for EFA between 132-726ng/band and for EMT & TEN between 132-396 ng/band, respectively. The correlation coefficient values were > 0.995 (n = 3). Typically, the regression equations for the calibration curve was found to be y = 3.919x +67.08 for EFA, y = 7.042x +93.60 for EMT and y = 5.853x +84.30 for TEN. The calibration curve was plotted by considering the peak areas versus corresponding concentration.

Sensitivity:

In present study the sensitivity parameter was evaluated by determining the LOD and LOQ of the drug. The LOD and LOQ parameter was evaluated by using the slope of line and standard deviation obtained from calibration curve studies.

Specificity:

Method specificity was evaluated for interference of closely related impurities and excipients in the analysis of drug solution. No change in R_f values on TLC plate was observed, which shows non interference of impurities and excipients and hence the method was found to be specific.

Accuracy:

To check the accuracy of the method, recovery studies were carried out at three different levels 80, 100 and 120%. Base level conc. of analytes in tablet formulation used were 300 ng/band, 150 ng/band and 200 ng/band for EFA, EMT and TEN, respectively.

Precision:

The method was assessed for precision by considering the results obtained for intra-day and inter-day variations while performed by the same analyst. Three replicates of three different concentrations were used for precision studies.

Robustness:

In present study it was evaluated by performing test on 3 different plates using same applied volume. The areas of the respective peaks of EFA, EMT, TEN remained constant.

Forced degradation studies:

Using acid hydrolysis, alkali hydrolysis, oxidation, thermal degradation and neutral degradation forced degradation studies were performed. All stress studies were performed to a mixture containing 100 ng, 150 ng and 300 ng/µl of EMT, TEN and EFA, respectively. Acid and alkali hydrolysis were carried out in 1N HCl and 1N NaOH, separately. The mixture was kept at 100^0C for 1 hr on water bath. For oxidative degradation, 3 ml of 3% H_2O_2 added and mixture was kept at 100 ^{0}C for 1 hr on water bath. For neutral stress study, solution of mixture was prepared in distilled water and kept at 100ºC for 1 hr. For thermal degradation tablet powder was left at 100^0 C for 24 hrs. The sample was then treated to obtain solution containing 600µg, 150µg and 100µg/ml of EFA, TEN and EMT, respectively.

RESULT AND DISCUSSION

The method development and validation of efavirenz, emtricitabine and tenofovir using HPTLC was carried out according to ICH guidelines to achieve simultaneous estimation of each drug in

bulk and multi component formulation and also applicable to analysis of the drug and degradation products in stability samples in industry for routine quality control analysis.

The mobile phase consisted of chloroform and methanol (9 mL: 1 mL v/v)) gave high resolution with R_f value 0.15, 0.34, 0.55 for emtricitabine, tenofovir and efavirenz, respectively. Calibration curve was obtained in the range of 132-726ng/spot, 132-462 and 132-462 ng/spot for efavirenz, emtricitabine and tenofovir, respectively. The regression coefficient was 0.998, 0.997 and 0.996 for efavirenz, emtricitabine and tenofovir, respectively. Accuracy parameter was checked by percent recovery study and found in the range i.e. 98-102% for each drug. Limit of detection and quantification was found to be 2.47 and 7.501ng/spot for efavirenz, 1.86 and 6.21 ng/spot for emtricitabine and 2.74 and 9.14 ng/spot for tenofovir, respectively. The precision at intraday and interday level was evaluated in terms of % RSD and was found to be less than 2%. The results of assay were in the range of 98.40% to 101.93% with 1.199, 1.144 and 1.115 as the values of standard deviation for EFA, EMT and TEN, respectively, which are shown in Table 1.

Table 1: Analysis of VIRADAY tablet formulation by HPTLC

Drug	Label claim (mg)	Mean amount found(mg ± SD)	% Label claim (% ± % RSD)
EFA	600	600.7 ± 1.201179	100.115±1.199
EMT	200	200.2 ± 1.145426	99.7 ±1.144
TEN	300	302.06 ±1.123079	100.6867±1.115

**n = 6, RSD = Relative standard deviation*

Percent degradation

Degradation was found to occur under acidic, basic, thermal stress and to a lesser extent, under oxidative stress but the drugs was stable to neutral stress. The assay of stressed sample was calculated against reference standards. Forced degradation studies gives pattern of degradation in different stress condition. The method was validated for accuracy, precision, specificity and linearity and range, the results obtained are depicted in Table 2. Values for LOQ and LOD are shown in Table 2.

Table 2: Linear regression data for calibration curve, LOD and LOQ

Drug	EFA	EMT	TEN
Linearity and range	132-726 ng/band	132-396 ng/band	132-396 ng/band
Slope (m)	3.919	7.042	5.853
Intercept (b)	67.08	93.60	84.30
Regression coefficient (r^2)	0.998	0.997	0.996
Limit of detection	2.47 ng/band	1.86 ng/band	2.74 ng/band
Limit of quantitation	7.501 ng/band	6.21 ng/band	9.14 ng/band

*n = 3

The recovery studies were found in the range i.e. 98-102%. The results obtained are depicted in Table 3. The % RSD was found to be < 2. are shown for EFA, EMT and TEN in Table 4. The

results of robustness studies obtained are shown in Table 5. In forced degradation studies, degradation pattern of each drug is shown in Table 6.

Table 3: Accuracy of the analysis of EFA, EMT and TEN in VIRADAY tablets.

Drug	% Level	Amount of tablet powder (ng/band)	Amount of bulk drug added (ng/band)	% Mean recovery ± %RSD
EFA	80	300	240	100.07± 0.073
	100	300	300	99.87 ± 0.12
	120	300	360	99.68± 0.178
EMT	80	100	80	99.70 ± 0.37
	100	100	100	100.4 ± 0.39
	120	100	120	99.46 ± 0.472
TEN	80	150	120	99.55 ± 0.189
	100	150	150	100.22 ± 0.425
	120	150	180	99.77 ± 0.458

*n = 9, %RSD = Percent relative standard deviation

Table 4: Intra-day and Inter-day precision study of the method for EFA, EMT and TEN

Drug	Theoretical con. (ng/spot)	Mean calculated con. (ng/spot) ± % RSD	
		Intra-day	Inter-day
EFA	132	130.993 ± 1.55	131.616 ±0.78
	330	331.096 ± 0.398	332.53± 0.21
	594	594.836 ± 0.248	591.396 ±0.10
EMT	132	133.576 ± 0.82	135.316 ±1.61
	264	263.22 ± 0.95	268.43 ± 0.41
	396	397.553 ± 0.88	398.41 ± 0.92`
TEN	132	132.056±1.17	134.43±0.59
	264	263.596±0.68	261.7±0.17
	396	397.583±0.54	399.42±0.24

*n = 9, RSD = Relative standard deviation

Table 5: Robustness study on HPTLC

Plates	Standard Concentrations (ng/spot)					
	EFA	TEN	EMT	EFA	TEN	EMT
	132	132	132	132	132	132
1	133.33	130.34	132.32	131.32	134.56	130.1
2	129.53	132.45	134.41	135.54	135.21	134.54
3	130.12	133.38	134.0	133.33	132.43	132.78
Mean	130.993	132.056	133.576	133.39	134.066	132.473
S.D.	2.04	1.55	1.1	2.11	1.45	2.23
% RSD	1.55	1.17	0.82	1.58	1.08	1.68

*n = 3, RSD = Relative standard deviation

The results of assay were in the range of 98.40% to 101.93% with 1.199, 1.144 and 1.115 as the values of standard deviation for EFA, EMT and TEN, respectively. A typical HPTLC chromatogram of EFA, TEN and EMT is depicted in Figure 2. Typical chromatogram for different types of stressed condition is depicted in Figure 3.

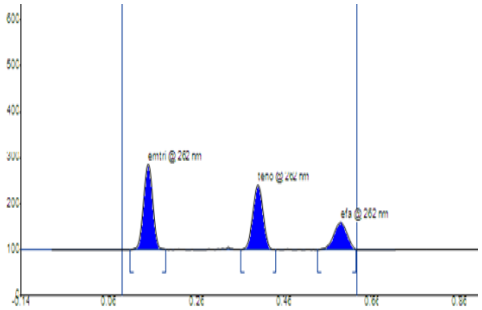


Figure 2: A typical HPTLC chromatogram of EFA and TEN and EMT for tablet powder analysis.

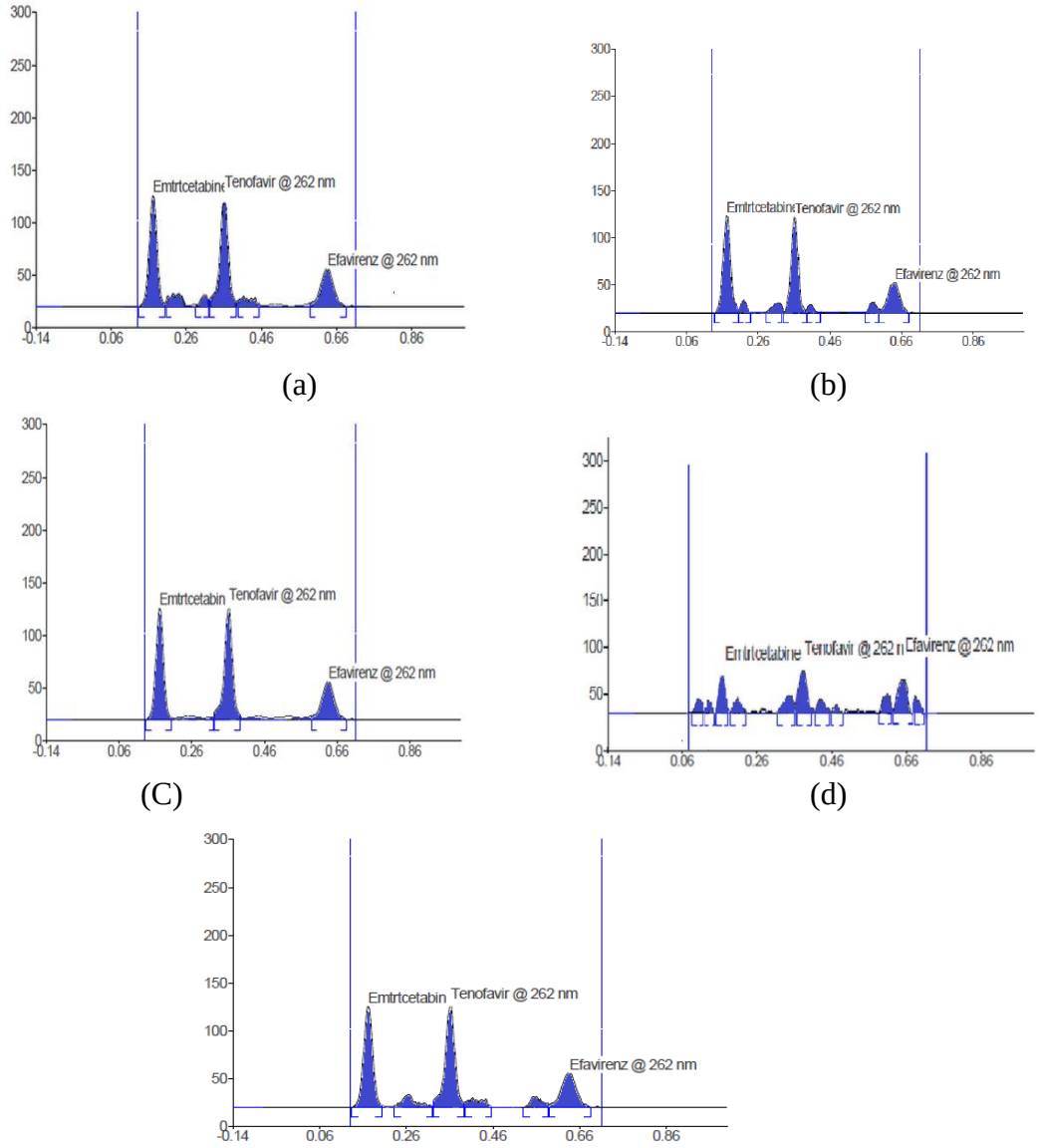


Figure 3: A typical HPTLC chromatogram of EFA and TEN and EMT fo (a) Acid hydrolysis, (b) Alkali hydrolysis, (c) Neutral hydrolysis (d) Thermal degradation, (e) Peroxide degradation

Table 6: Summary of forced degradation studies on HPTLC

Exposure Condition	Efavirenz R_f of deg. peaks	% deg.	Emtricitabine R_f of deg. peaks	% deg.	Tenofovir R_f of deg. peaks	% deg.
Acid, 1N HCl, 100 ^{0}C ,1 hr	No extra peak	0.00	0.24	14.14	0.30, 0.42	19.20
Base, 1N, NaOH, 100 ^{0}C,1hr	0.60	12.67	0.23	9.02	0.29, 0.40	20.31
H_2O_2 (3%,v/v) 4 hrs	0.59	10.41	No extra peak	0.00	0.26, 0.43	17.06
Thermal,100^{0}C 24 hrs	0.61, 0.68	22.32	0.11, 0.13, 0.23	43.19	0.30, 0.39	37.33
Neutral hydrolysis	No extra peak	0.00	No extra peak	0.00	No extra peak	0.00

R_f of deg. peaks = R_f value of degradation peak, % deg.

CONCLUSION

The developed HPTLC method was found to be accurate, simple, precise, specific and stability-indicating and can be conveniently applied for quality control analysis in industry and is having short run time which significantly reduces the analysis time and cost.

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