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Development and Validation of HPTLC Method for Simultaneous Estimation of Emtricitabine, Rilpivirine and Tenofovir Disoproxil Fumarate in Combined Dosage form

J. Saminathan and T. Vetrichelvan

Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, Melmaruvathur-603 319 Kanchipuram Dist, Tamil Nadu, India

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Abstract

This study describes the development and validation of high performance thin layer chromatographic (HPTLC) method for the simultaneous estimation of Emtricitabine (EMT), Rilpivirine (RPV) and Tenofovir disoproxil fumarate (TFV) in combined dosage form. Chromatographic separation of these drugs was performed on aluminum plates precoated with silica gel 60 F_{254} as the stationary phase using solvent system consisted of chloroform: ethyl acetate: methanol: glacial acetic acid (5:2:1:0.1 v/v/v/v). The densitometric analysis was carried out in absorbance mode at 272 nm. The drugs were satisfactorily resolved with R_f values of 0.28 ± 0.02, 0.70 ± 0.02 and 0.52 ± 0.04, respectively. The method was validated according to the International Conference of Harmonization (ICH) guidelines. The calibration curves were linear over the (r^2 > 0.999) concentrations range from 600-2400 ng $band^{-1}$ for Emtricitabine, 50-300 ng $bands^{-1}$ for Rilpivirine and 600-3600 ng $band^{-1}$ for Tenofovir disoproxil fumarate. The method showed accuracy of 100.01%, 100.32% and 100.14% and percentage assay of 99.91%, 98.72% and 99.34% for Emtricitabine, Rilpivirine and Tenofovir disoproxil fumarate, respectively. Percentage relative standard deviation (<2%) was found for both precision and robustness study showing that the proposed method was precise, specificity, robust and stable in accordance with ICH guidelines.

Key words: Emtricitabine, rilpivirine, tenofovir disoproxil fumarate, HPTLC, validation

Introduction

Complera, a combination drug of EMT, RPV and TFV, is used as a complete single-tablet regimen to treat HIV-1 infection in adults. United States Food and Drug Administration (FDA) approved for use in people with HIV who have not previously been treated with antiretrovirals. The newest combination tablet offers complete HIV drug coverage in a single daily dose. EMT, chemically 5-fluoro-1-(2*R*, 5S)-[2 (hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (Figure 1a) is nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). RPV is chemically known as 4-[[4-[[4-[(*E*)-2-cyanoethenyl]-2, 6 dimethyl phenyl] amino] 2 pyrimidinyl] amino] benzonitrile monohydrochloride (Figure 1b). It is a diarylpyrimidine derivative, a potent non nucleoside reverse transcriptase inhibitor (NNRTI) of the human immunodeficiency virus type 1 (HIV-1). TFV is 9[(*R*) 2[[bis [[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate (Figure 1c). Both EMT and TFV are included in the official books of United States Pharmacopoeia 2005 and Indian Pharmacopoeia 2014 except Rilpivirine is not official in any of the pharmacopoeias like IP, BP and US Pharmacopoeia. Literature survey reveals that there is no reported HPTLC method for simultaneous determination of EMT, RPV and TFV (Ahindita *et al.,* 2011; Anandakumar *et al.,* 2011; Choudhari *et al.,* 2013; Choudhari *et al.,* 2010; Ghorpade *et al.,* 2010; Ilango *et al.,* 2012; Jayakar *et al.,* 2010; Patel *et al*., 2009; Sudha *et al.,* 2010) HPLC (Murali *et al.,* 2011; Pranitha *et al.,* 2012; Rao *et al.,* 2011; Rezk *et al.,* 2005) HPTLC (Joshi *et al.,* 2009). The present study was to develop accurate, precise and

Correspondence to: J. Saminathan; E-mail: swamilingam@gmail.com

sensitive HPTLC method and to validate as per International Conference on Harmonization of Technical Requirements for registration of Pharmaceuticals for human use guidelines (ICH 2005).

Materials and Methods

Materials and reagents: Pharmaceutical grade EMT, RPV and TFV working standard were obtained as a gift samples from Strides Arcolabs Ltd., Bengaluru, India. The commercially available tablet Complera® containing a combination of EMT- 200 mg, RIL-25 mg and TFV-300 mg were procured from Gilead sciences. Analytical grade methanol (Qualigens, India Ltd.), chloroform (Qualigens, India Ltd.) and ethyl acetate (Merck Specialities Private Ltd., Mumbai) were used in the present study.

Equipments and chromatographic conditions: The method development was performed by using Camag HPTLC containing Camag Linomat 5 S/NO.180750 applicator, Hamilton 100 microlitre sample syringe, E. MERCK KGaA silica gel *(*Art. No.1.05554.0007*)* precoated plate 60 F 254, [(20 × 10 cm) with 250 µm thickness; supplied by Anchrom Techno, Mumbai]. The plates were prewashed with methanol and activated at 120 ºC for 20 min prior to chromatography. A constant application rate of 0.1 μlS^{-1} was used and the space between two bands was 6 mm. The slit dimension was kept at 4 mm × 0.30 mm and the scanning speed was 20 mmS^{-1}. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ºC ± 2). Densitometric scanning was performed using a Camag TLC scanner III (Camag, Muttenz, Switzerland) in the reflectance-absorbance mode and operated by winCATS software (V1.4.6). The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 200 and 800 nm. Dissolution of the compounds was enhanced by sonication on a Shimadzu sonicator and REMI centrifuge. The mobile phase was consisted of chloroform: ethylacetate: methanol: glacial acetic acid (5:2:1:0.1, v/v/v/v) and 10 ml of the mobile phase was used for chromatography. All the drugs showed considerable absorbance at 272 nm. So, 272 nm was selected as the wavelength for detection overlain spectra shown in figure 2.

Preparation of standard stock solution and calibration curve: Standard stock solutions to get a concentration of 2 mg/ml, 0.25 mg/ml and 3 mg/ml solution were prepared by using methanol as a diluent. The mixed standard solutions were prepared by dilution of the stock solution with mobile phase to give concentrations ranging from 400-2400 ng/band of EMT, 50-250 ng/band of RPV and 600-3600 ng/band of TFV.

Preparation of sample preparation: Twenty Complera® tablets (each tablet contained 200 mg of EMT, 25 mg equivalent of RPV and 300 mg of TFV were weighed, powdered and average weight was calculated. Tablet powder equivalent to 30 mg of TFV was transferred in to a 100 ml volumetric flask. The drug was extracted by addition of methanol with shaking and the solution was filtered through Whatman filter paper (No.14). The volume was then made to the mark with methanol and filtered to obtain a concentration of 0.3 mg/ml, 0.025 mg/ml and 0.2 mg/ml. The formulation was assayed by spotting 2.5 µl of the solution on to the plate followed by development and scanning HPTLC densitogram obtained from the sample solution shown in figure 3. Six determinations were performed.

Results and Discussion

Method development and optimization: Different mobile phases containing chloroform, hexane, toluene, methanol, ethyl acetate, formic acid and glacial acetic acid used in different proportions were examined. Chloroform: methanol: ethyl acetate: glacial acetic acid 5:2.1:0.1 (v/v/v/v) was finally selected because it resulted in acceptable resolution of the bands with R_f values of 0.28 ± 0.02 for EMT, 0.71 ± 0.02 for RPV and 0.50 ± 0.01 for TFV.

Method validation

The method was validated as per ICH guideline parameters ICH Q2A (R1) 2005) used for the assay of a dosage form such as linearity, precision, accuracy, specificity quantification limit, detection limit and robustness.

Linearity (plotting of calibration graph): Linearity of the method was studied by taking six calibration points for Emtricitabine, Rilpivirine and Tenofovir disoproxil fumarate. The mixed standard solutions in the concentration range of 400 - 2400 ng/band of Emtricitabine, 50 - 300 ng/band of Rilpivirine and 600 - 3600 ng/band of Tenofovir disoproxil fumarate injected 6 times into the CAMAG HPTLC system keeping the injection volume constant with correlation coefficients of 0.9998 ± 0.002, 0.9994 ± 0.0055 and 0.9991 ± 0.0027 shown in the table 1. Calibration curve of emtricitabine, rilpivirine and tenofovir disoproxil fumarate were shown in figure 4. Plots of residuals against the concentrations of drugs showed the residuals were distributed both above and below the zero residual line shown in figure 5.

Table 1. Method validation parameters of Emtricitabine, rilpivirine and Tenofovir disoproxil fumarate.

Parameters	Emtricitabine	Rilpivirine	Tenofovir disoproxil fumarate
Detection wavelength	272 nm	272 nm	272 nm
Linearity range (ng/band)	400-2400	50-300	600-3600
Linearity equation	y=6.5963x+239.51	y=50.5197x+64.8098	y=3.9349x+219.92
Slope	6.5963	50.5197	3.9349
Intercept	239.51	64.8098	219.92
Correlation coefficient r	0.9992 ± 0.002	0.9995 ± 0.0055	0.9993 ± 0.0027
Standard deviation, n=6	0.52	1.12	0.68
Limit of detection (ng/band)	5.0164	4.2500	5.5063
Limit of quantification ng/band)	15.2012	12.8790	16.6890

Table 2. Assay results of fixed dose combined tablets (n=6).

Parameters	Emtricitabine	Rilpivirine	Tenofovir
Label claim (mg/tab)	200 mg	25 mg	600 mg
Actual amount added (ng/band)	1000 ng	125 ng	1500 ng
Amount obtained (ng/band ± SD)	992.93 ± 4.97	123.66 ± 1.97	1496.83±3.86
drug content	99.29 ± 0.48	98.92 ± 1.25	99.78 ± 0.81
% RSD	0.5008	1.1380	0.8120

Table 3. Intra and inter-day precision for emtricitabine, rilpivirine and Tenofovir disoproxil fumarate (n=6).

Drug	Amount labeled (mg/tab)	Percentage obtained*		SD		% RSD	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
Emtricitabine	200	99.58	99.56	0.5356	0.2967	0.5346	0.2970
	200	100.35	100.12				
	200	100.61	100.01				
Mean		100.18	99.89				
Rilpivirine	25	98.96	99.15	0.4981	0.6630	0.5004	0.6656
	25	99.78	100.37				
	25	99.86	99.31				
Mean		99.53	99.61				
Tenofovir Disoproxil fumarate	300	100.13	99.56	0.2042	0.3650	0.2035	0.3656
	300	100.51	99.67				
	300	100.45	100.24				
Mean		100.36	99.82				

Table 4. Recovery study for EMT, RPV and TFV (n=3).

Drug	Amount added (ng/band)	Amount estimated (ng/band)*	Amount recovered (ng/band)	% Recovery	S.D	% RSD
EMT	800	1800.197	807.2555	100.90		
	1000	2001.925	1008.9835	100.89		
	1200	2208.861	1215.9195	101.32	0.2454	0.2429
			Mean	**101.03**		
			C.I	**100.42-101.63**		
RPV	100	225.649	101.9805	101.98		
	125	252.091	128.4225	102.73		
	150	274.653	150.9845	100.65	1.0533	1.0349
			Mean	**101.78**		
			C.I	**99.16-104.39**		
TFV	1200	2706.141	1209.362	100.78		
	1500	3004.033	1507.254	100.48		
	1800	3304.642	1807.863	100.43	0.1892	0.1882
			Mean	**100.56**		
			C.I	**100.09–101.03**		

[1]Average value ± relative standard deviation from three analyses

Table 5. Effect of mobile phase composition and volume variation on R_f values.

Mobile phase composition, v/v/v/v chloroform: ethyl acetate: methanol: glacial acetic acid	R_f value		
	EMT	RPV	TFV
5.0:2.0:1.0:0.1 (optimized)	0.28	0.70	0.52
5.0:1.5:1.5:0.1	0.26	0.67	0.47
5.0:1.5:2.0:0.1	0.27	0.65	0.51
5.0:2.0:1.5:0.1	0.29	0.66	0.51
4.5:2.5:1.0:0.2	0.32	0.67	0.49
4.5:1.5:1.5:0.1	0.29	0.72	0.51

Table 6. Peak area Robustness study for the developed method (n=6).

Parameter studied	% RSD		
	EMT	RPV	TFV
Composition of mobile phase(±2%)	0.98	1.31	1.27
Volume of mobile phase (±5%)	1.34	1.29	1.38
Time from spotting to development (10 min)	0.61	0.87	0.72
Time from development to scanning (10min)	0.68	0.74	0.83

* % RSD were calculated from the peak areas of densitograms

Table 7. Stability of the plate.

Volume applied	Time in hours	Peak area		
		EMT	RPV	TFV
2.5 µl	0	4672.64	4922.31	4277.17
	1	4642.12	4909.36	4249.73
	2	4654.34	4914.83	4264.22
	4	4606.48	4894.97	4237.34
	7	4622.72	4889.23	4228.39

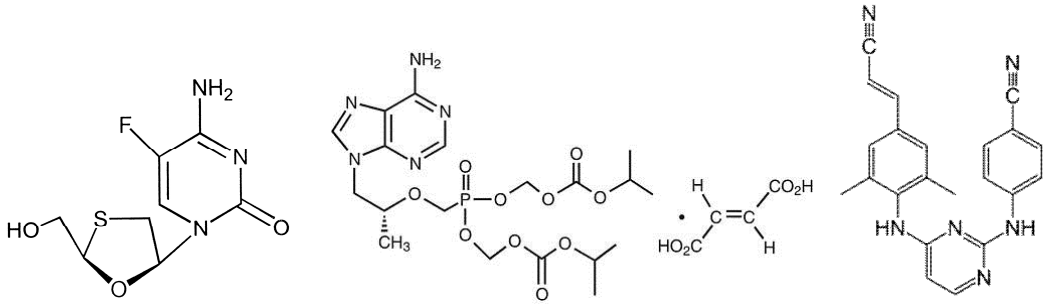

Figure 1. Chemical structure of EMT, RPV and TFV.

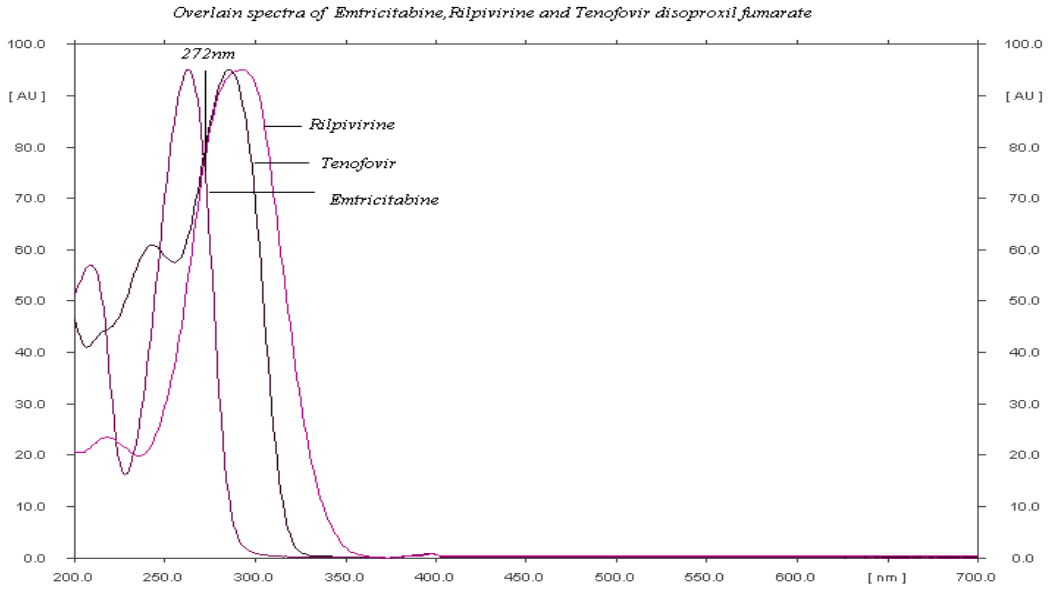

Figure 2. Overlain spectra of EMT, RPV and TFV.

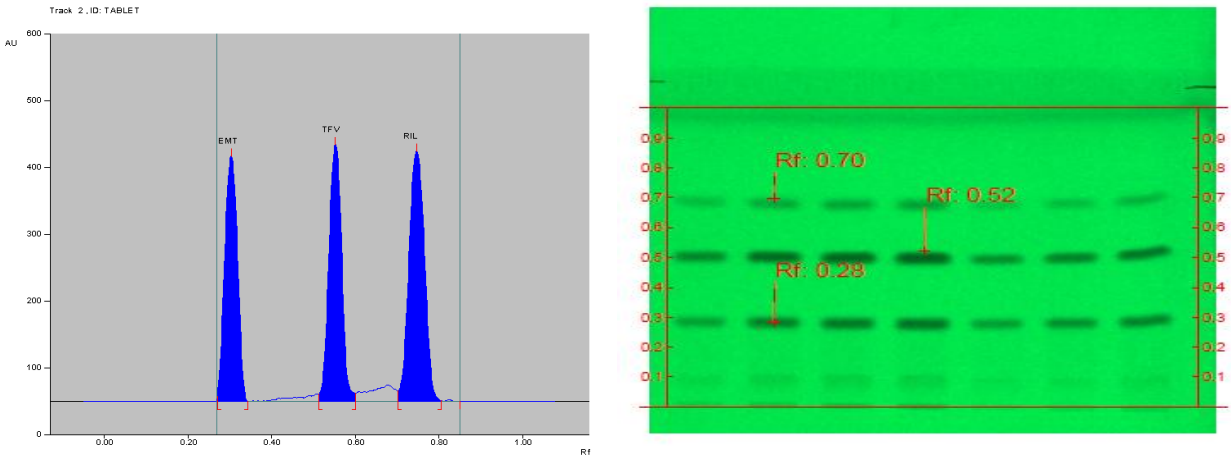

Figure 3. HPTLC densitogram obtained from the sample solution of EMT (1000 ng/band, R_f = 0.28 ± 0.02), RPVe (125 ng/band, R_f = 0.70 ± 0.02) and TFV (1500 ng/band, R_f = 0.52 ± 0.04) @ 272 nm.

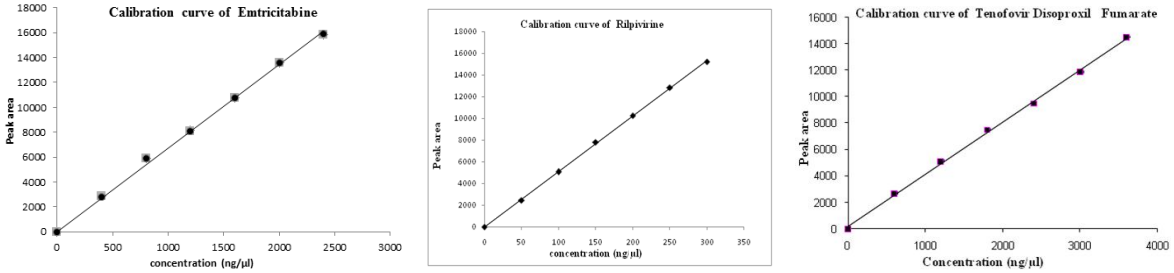

Figure 4. Calibration curve of EMT, RPV and TFV.

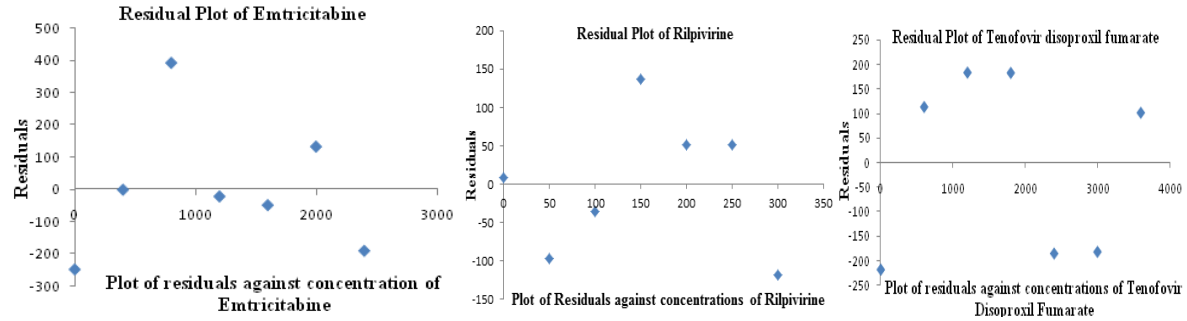

Figure 5. Plot of residuals against various concentrations.

Precision: To study the precision, Intra-day and Inter-day precision was studied by taking three different concentrations 800, 1200 and 1600 ng/band of EMT, 100, 150 and 200 ng/band of RPV and 1200, 1800 and 2400 ng/band of TFV and three replicates of each concentration to see the variation of their peak area within a day and for three different days. Six replicate analyses were performed on accurately weighed amounts of the tablets. The assay (%) was found to be 99.29 ± 0.48 for EMT, 98.92 ± 1.25 for RPV and 99.78 ± 0.81 for TFV, with % RSD values of 0.50, 1.59 and 0.25, respectively shown in table 2. Intra-day Precision, as RSD (%), was found to be 0.56 for EMT, 0.56 for RPV and 0.81 for TFV, with standard error of 0.34, 0.24 and 0.56, respectively. Interday Precision, as RSD (%), was found to be 0.80 for EMT, 0.80 for RPV and 1.90 for TFV, with standard error of 0.43, 0.42 and 1.09 shown in table 3.

Accuracy: To study the recovery of formulation, standard drugs of EMT, RPV and TFV at 80%, 100%, 120% were added to the labeled claim of EMT 200 mg (i.e. the spiked amounts were 800, 1000, 1200 ng/band). To study the recovery of RPV, standard were added to the labeled claim equivalent of RPV 25 mg (i.e. the spiked amounts were 80, 100 and 120 ng/band). Similarly, to study recovery of TFV, standards were added to the labeled claim of TFV 300 mg (i.e. the spiked amounts were 1200, 150, 1800 ng/band). For EMT, % recovery ranged from 100.01 ± 0.42%, with 95% confidence interval ranging from 98.95 – 101.06%. For RPV, % recovery ranged from 100.32 ± 0.48%, with 95% confidence interval ranging from 99.12 – 101.51. For TFV, % recovery ranged from 100.14 ± 0.05%, with 95% confidence interval ranging from 99.99-100.28% found to be within the limits shown in table 4.

Limit of detection and quantification: Determinations of limit of detection and quantification were based on the standard deviation of the response and the slope as:

$$LOD = 3.3\ \sigma/S, \text{ and } LOQ = 10\sigma/S$$

Where σ is the standard deviation of y-intercepts of regression line and S is the slope of the corresponding standard curve. LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for EMT, RPV and TFV by spotting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The

LOD and LOQ were 5.0164 and 15.2012 ng/band for EMT, 4.25 and 12.8790 ng/band for RPV and 5.5063 and 16.6890 ng/band for TFV shown in table 1.

Specificity: The specificity of the method was confirmed by comparing the R_f values and spectra of the spots with that standards and test samples. The peak purity of samples was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot. The peak purity was determined on WinCATS software V 1.4.6. Acceptable peak purity and correlation values suggest no interference in the quantification of analyzed drugs in sample solutions. This proves that the method were specific.

Robustness: The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Small changes in the mobile phase composition (± 0.1 ml), the effect on the results were examined. Concentrations of 800 ng/band for EMT, 100 ng/band for RPV and 1200 ng/band for TFV were applied to study the robustness of the method. The effect of these changes on R_f values and peak area was evaluated by calculating the relative standard deviations (RSD) for each parameter. Mobile phases having different proportions of components, e.g. chloroform: ethylacetate: methanol: glacial acetic acid in the ratio of 5:1.5:1.5:0.1 v/v/v/v, 5:1.5:2:0.1 v/v/v/v, 5:2.0:1.5:0.1 v/v/v/v, 4.5:2.5:1:0.2, 4.5:1.5:1.5:0.1 v/v/v/v etc., were tried shown in table 5. The time from spotting to chromatography and from chromatography to scanning was varied by 10 min and analysed. The robustness of the method was determined at different proportions of mobile phase. The effect of changes on R_f values and peak area was evaluated by calculating the relative standard deviations (RSD) for each parameter. The peak areas were unaffected (RSD < 2%) by small changes of the operating conditions. The results from the robustness study listed in table 6.

Stability study: The stability of the drugs on the TLC plates, the freshly prepared drug solutions were applied to the plates and developed at a room temperature. The sample solutions were spotted at initial, 1 hr, 2 hr, 4 hr and 7 hrs. No decomposition of the drug was observed during densitogram development. No significant decrease in peak area was found for a stock solution after storage at room temperature for 7 hours. These observations suggest that all the drugs were stable for 7 hrs shown in table 7.

Conclusion

The validated HPTLC method employed here proved to be simple, rapid, accurate, precise and robust and can thus be used for routine analysis of EMT, RPV and TFV in a combined tablet dosage form.

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