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A NEW RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF EMTRICITABINE AND TENOFOVIR IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A new, simple and rapid RP-HPLC method was developed and validated for the estimation of EMTRICITABINE and TENOFOVIR in pharmaceutical dosage forms. The chromatographic separation was achieved on SHISEIDO C18 column (250 x 4.6 mm i.d, 5µ) using Methanol: 20nM phosphate buffeer (pH 5.0±0.05) in the ratio of 35:65v/v, with a flow rate of 1 ml/min and detection at 271 nm. The retention times for EMT and TEN were found to be 2.80 and 4.81 min respectively. Linearity was established in the range of 10-60µg/ml for both EMT and TEN respectively. The method was precise with %RSD < 2 for both intraday and interday precision. The accuracy of the method was performed over three levels of concentration and the recovery was in the range of 98-102%.

KEYWORDS: *Emtricitabine, Tenofovir, RP-HPLC, Method development, Validation*

INTRODUCTION

Emtricitabine is an antiretroviral medication that belongs to the class of nucleoside reverse transcriptase inhibitors (NRTIs), commonly used in the treatment of HIV infection. It is a cytidine analog that, once inside the body, is converted into its active form, emtricitabine triphosphate. This active metabolite works by inhibiting the reverse transcriptase enzyme, which is essential for the virus to convert its RNA into DNA. Additionally, it gEMT incorporated into the growing viral DNA chain, leading to premature chain termination. As a result, the replication of HIV is effectively suppressed. Tenofovir is an antiretroviral medication that belongs to the class of nucleotide reverse transcriptase inhibitors (NtRTIs), a subgroup of nucleoside reverse transcriptase inhibitors (NRTIs), and is widely used in the treatment of HIV infection as well as chronic Hepatitis B. It is an analog of adenosine monophosphate and, after entering the body, is converted into its active form, tenofovir diphosphate. This active metabolite works by inhibiting the reverse transcriptase enzyme required for viral replication. It competes with natural nucleotides and gEMT incorporated into the viral DNA chain, causing premature chain termination. As a result, viral DNA synthesis is halted, preventing the virus from multiplying effectively.

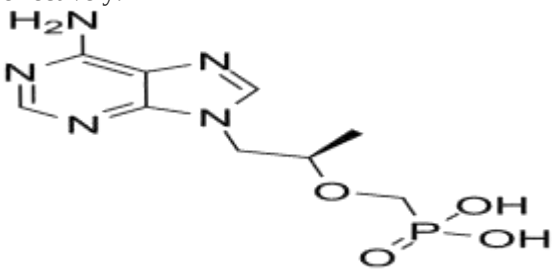

Fig.1 Chemical structure of Tenofovir

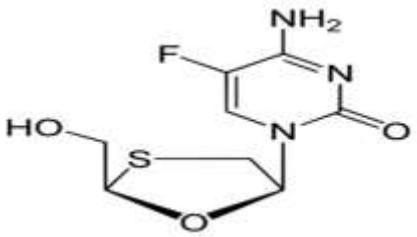

Fig.2 Chemical structure of Emtricitabine

The present study is to develop a simple and rapid RP-HPLC method for EMT and TEN. A Literature survey reports that analytical methods for the estimation of EMT and TEN based on UV [4-8], HPTLC [9], HPLC [10-17], Stability indicating HPLC [18-20], related substances [21-24], LC-MS [25] were reported.

Although different analytical methods are available, there are very few methods developed on simultaneous estimation of emtricitabine and Tenofovir in pure drugs. The main objective of the study is to develop and validate a cost effective, accurate and precise method for the estimation of EMT and TEN in bulk and pharmaceutical formulations.

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MATERIALS AND METHODS

A. Chemicals and reagents

Emtricitabine (EMT) and Tenofovir (TEN) working standards were procured from Yarrow Chemicals Pvt. Ltd, Mumbai. Commercially available as Ricovir-em were procured as gift samples from Mylan Pvt. Ltd. HPLC grade water was purchased from Thermo Fisher Scientifics Ltd., Mumbai. HPLC grade methanol, Acetonitrile, Orthophosphoric acid, Acetic acid, Triethyl amines, Potassium hydroxide of AR grade were procured from Merck specialties Pvt. Ltd., Mumbai.

B. Instrumentation and analytical conditions

RP-HPLC method was performed on the HPLC system (Shimadzu) consisting of binary gradient pump with UV detector (LC-20AD). Rheodyne injector with 20 µl fixed loop was used for injecting sample on SHISEIDO C18 column (250 x 4.6 mm i.d, 5µ) in the present study.

C. Preparation of solutions

i. Preparation of standard stock solutions

Standard stock solutions were prepared by transferring accurately weighed 100 mg of EMT, TEN into separate 100 ml volumetric flask and make up to required volume with HPLC grade water for EMT and Acetonitrile for TEN. From this take 1ml and make up to 10ml this gives the conc. 100ug/ml. Finally dissolved in diluent (mobile phase) to obtain a standard solution of EMT (10 µg/ml), and TEN (10 µg/ml)

ii. Preparation of the mobile phase:

The mobile phase is a mixture of methanol and ortho phosphate buffer, p^H is adjusted to 5.0 using ortho phosphoric acid. The prepared mobile phase was filtered through 0.45 µm membrane filter (Millipore) and sonicated before use. Mobile phase is pumped in the ratio of 35: 65 %v/v (Methanol : Ortho phosphate buffer).

RESULTS AND DISCUSSION

A. Method development and optimization

The choice of the detection wavelength was based on the scanned absorption spectrum of Emtricitabine and Tenofovir. 10 mg of Emtricitabine and Tenofovir were dissolved in 10 ml of methanol.The UV-spectrum of Emtricitabine and Tenofovir was separately scanned in the wavelength range 200-400 nm against blank. After correlation of the spectrums 271 nm wavelength was selected for the analysis (Fig. 3). Trails were performed using different columns (Hypersil BDS C18, Symmetry C18, Phenomenex C18 and Shiseido C18), buffers (Acetate, Phosphate, Ortho phosphoric acid), pH (3-6), organic phases (Acetonitrile, Methanol). Shiseido C18 column (250mm X 4.6 mm, 5 µ) produced good separation with efficient resolution and more theoretical plates. The drugs were eluted with Shiseido C18 column at a flow rate of 1.0 ml/min using a mobile phase consisting of methanol: ortho phosphate buffer (pH 3.0) in the ratio of 35:65%v/v respectively. The retention times for EMT and TEN were found to be 4.15 and 6.8 min respectively.

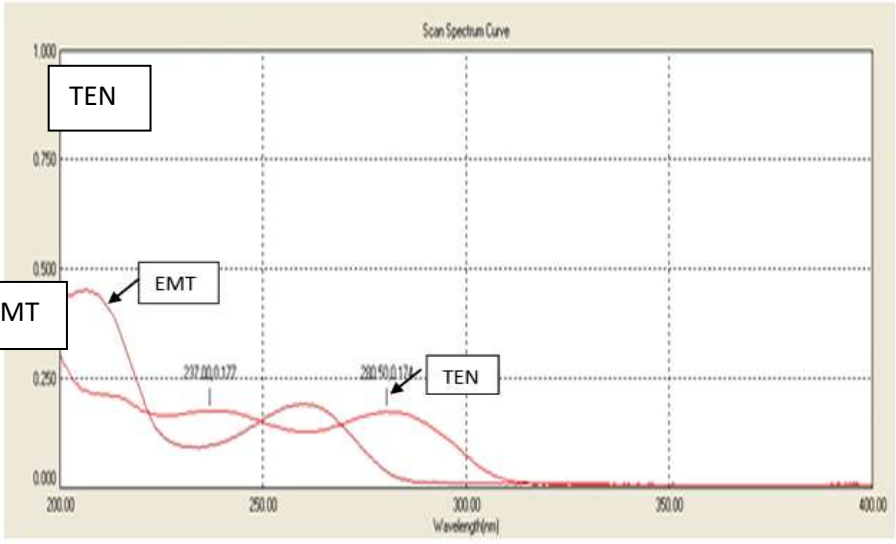

Fig.3: UV Overlay spectrum of EMT and TEN

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B. System Suitability

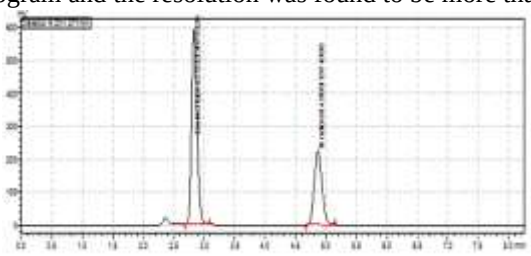
Under optimized chromatographic conditions 20 µl of solution containing 30 µg/ml of EMT and 30 µg/ml of TEN was injected into the system in six replicates. Chromatograms were recorded and studied for different system suitability parameters like retention time, peak area, number of theoretical plates, tailing factor and resolution. The results were shown in table 1.

Table 1. System suitability results for EMT and TEN

INJECTION	EMT peak area	TEN peak area
Injection1	36235	42297
Injection2	36245	42284
Injection3	36195	42285
Injection4	36225	42287
Injection5	36215	42279
Injection6	36238	42299
Average	36225.5	42288.5
Standard deviation	18.26198	7.842193
%RSD	0.050411	0.018544
Theoretical Plates	6412	5192
Tailing factor	1.1	1.2

C. Specificity

The HPLC chromatograms were recorded for blank (Fig. 4a) and standard (Fig. 4b) under optimized analytical conditions and compared for additional peaks, however no additional peaks were found. The two peaks were completely separated in HPLC chromatogram and the resolution was found to be more than 2.



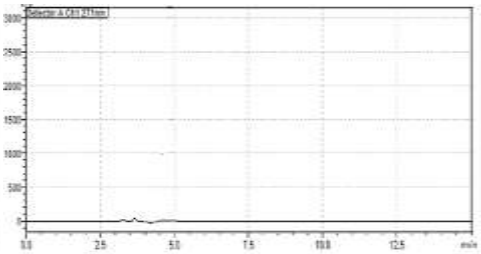


Fig. 4 a: Chromatograms for specificity of EMT **Fig 4b.Blank chromatogram And TEN**

D. Linearity

Linearity was established over the range of 10µg/ml to 50 µg/ml for both EMT and TEN by constructing calibration graph between the tested concentration level and corresponding peak areas for six determinations and the results were shown in table 2 and linearity graphs were shown as fig 5a, 5b. The correlation coefficients were >0.99 for two drugs, which meet the method validation acceptance criteria, and hence, the method is said to be linear for the drugs.

Table 2. Linearity results for EMT and TEN

	EMT		%RSD	TEN		%RSD
	Conc. (µg/ml)	Peak Area*±SD		Conc. (µg/ml)	Peak Area*±SD	
Blank	0	0.00	-	0	0.00	-
Level-1	0.5	20578±852.12	0.564	0.75	23247±945.25	0.542
Level-2	0.75	28082±729.12	0.485	1.25	34034±993.45	0.457
Level-3	1.0	36235±954.23	0.451	1.50	42297±845.28	0.417
Level-4	1.25	43191±784.65	0.428	1.75	48858±789.24	0.325
Level-5	1.5	54267±845.45	0.495	2.0	56133±684.79	0.517

*Mean of 6 determinations

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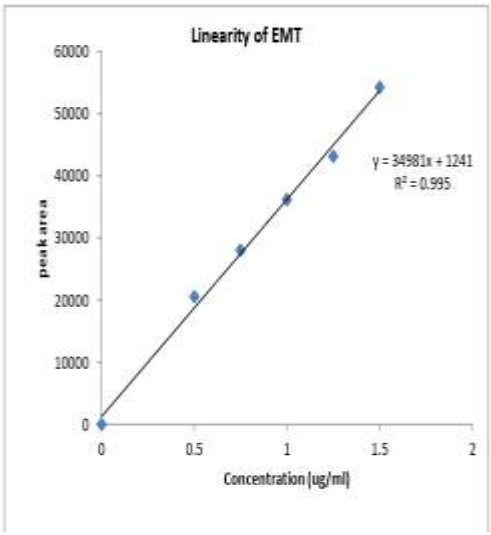

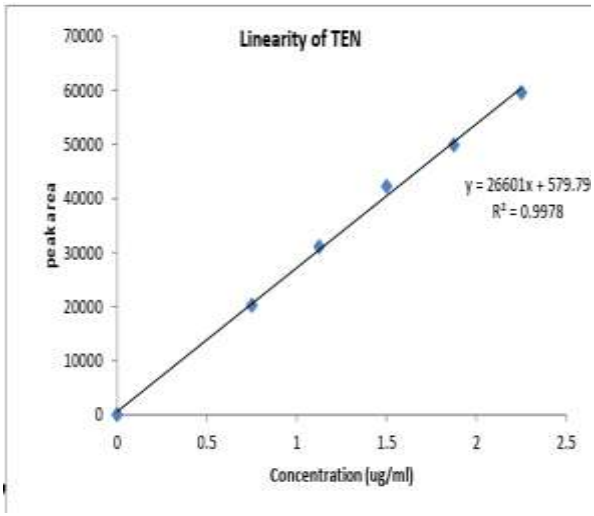

Fig. 5a: Linearity plot of EMT **Fig. 5b: Linearity plot of TEN**

E. Accuracy

The accuracy for proposed method was determined, recovery studies were performed in mentioned levels and recorded (Table 3), Obtained results were found to be within the limits of 98-102%, indicating an agreement between the true value and found value.

Table 3. Recovery studies of EMT and TEN

Drug	Conc. Std (µg/ml)	Conc. added (µg/ml)	Amount Recovered (µg/ml)	% Recovery* ±S.D.	%RSD
EMT	20	0.5	20.55	100.24±1715.119	0.718
	20	1.0	21.02	100.09±623.939	0.131
	20	1.5	21.56	100.27±8162.395	1.133
TEN	5	0.75	5.76	100.17±5957.928	0.494
	5	1.5	6.54	100.61±8219.499	1.793
	5	2.0	7.12	101.71±3023.633	0.419

*Mean of six determinations

F. Precision

Precision was calculated as intra-day and inter-day variations for the drugs. Percent relative standard deviations for estimation of EMT and TEN under intra-day and inter-day variations were found to be less than 2. Results were showed in Table 4.

Table 4. Precision values of EMT and TEN

Drug	Conc. (µg/ml)	Intra-day*		Inter-day*	
		Mean area*±S.D.	%RSD	Mean area*±S.D.	%RSD
EMT	0.5 (LQC)	18882.33±752.10	0.158	18866.00±188.33	0.397
	1.0 (MQC)	31941.67±450.67	0.473	32220.00±653.86	0.684
	1.5 HQC)	46328.00±1934.1	1.033	46635.67±516.26	0.394
TEN	0.75 (LQC)	21815.67±141.98	0.309	21664.67±862.76	1.825
	1.5 (MQC)	39287.00±733.32	0.763	39045.33±816.02	0.867
	2.0 (HQC)	52283.33±606.08	0.426	51834.33±2346.44	1.636

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G. Sensitivity

It is expressed as Limit of detection and Limit of quantitation. LOD is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) with a stated confidence level (generally 99%). LOQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met.

Table 5. LOD and LOQ of EMT and TEN

Parameter	EMT	TEN
LOD (µg/ml)	0.100	0.120
LOQ (µg/ml)	0.500	0.550

G. Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, ± 1% in the ratio of acetonitrile in the mobile phase, varying wavelength and ± 0.1 ml of the flow rate.

Table 6. Robustness Parameters of EMT and TEN

S.No	Flow rate (ml/ min)	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
1	Less Flow (0.9)	4.751	42103	0.20	2426.0	1.5
			42134			
			42145			
2	Actual Flow (1.0)	4.753	39152	0.29	2542.7	1.6
			38568			
			39864			
3	More Flow (1.1)	4.800	38912	0.15	2416.5	1.5
			38534			
			38563			

***Average of 3 determinations**

I. Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type.

Table 7. Results for ruggedness of EMT and TEN

Drug	Analyst	Retention time (min)	Peak Area	RSD (%)	System suitability results	
					Plate count	Tailing factor
EMT	Analyst 1	2.81	36542	0.03	3880	1.13
	Analyst 2	2.88	36485	0.05	3884	1.24
TEN	Analyst 1	4.77	42276	0.02	5097	1.12
	Analyst 2	4.79	42284	0.03	5091	1.05

J. Assay

The marketed formulation used was sylate-M, consists of 500 mg of Emtricitabine and 500 mg Tenofovir

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Table 8. Assay of EMT and TEN in pharmaceutical formulation

Drug	Label claim	Amount found	Mean* %Recovery ± S.D.	%RSD*
EMT	200 mg	199.79	99.89±0.692	0.693
TEN	245 mg	244.86	99.94±0.963	0.963

*Values are expressed as mean ±SD (n= 3)

CONCLUSION

In the present work a new, simple, accurate and rapid method was developed by using RP HPLC-UV to determine simultaneous estimation of Emtricitabine and Tenofovir. The method was validated according to ICH guidelines. Method validation was done by testing its linearity, accuracy, precision, values of LOD and LOQ. Compared to other methods, this RP HPLC method is simple as well as economic for the detection.

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