International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.5, pp 731-739, 2017

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Tenofovir Alafenamide and Emtricitabine in Bulk and Tablet Dosage Form

Bhushan P. Badgujar*, Moreshwar P.Mahajan, Sanjay D. Sawant.

Department of Quality Assurance Technique, Smt. KashibaiNavale College of Pharmacy Kondhwa (Bk.) Pune-411048, India.

Abstract : A simple, precise, reliable, rapid and reproducible reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the simultaneous estimation of Emtricitabine and Tenofovir alafenamide. Chromatography was carried out Younglin (S.K) Gradient System UV Detectoron C18(4.6X250 mm, 5µ) column with a mobile phase composed of Methanol: Distill water (60:40 v/v) at a flow rate of 1 ml/min. The pH of mobile was adjusted by 0.05% ortho phosphoric acid (pH-3). Detection was carried out using a UV detector at 260 nm.Parameters such as linearity, precision, accuracy, ruggedness, LOD and LOQ were studied as per the ICH Q2(R1) guidelines. The retention times of Emtricitabine and Tenofovir were 3.10 min and 7.38 min respectively. The linearity range for Tenofovir alafenamide and Emtricitabine were 5-30µg/ml, 40-240µg/ml respectively. The correlation coefficients of Emtricitabine and Tenofovir were found to be 0.999. Developed method was found to be accurate, precise, selective and rapid for simultaneous estimation of Emtricitabine and Tenofovir alafenamide in pharmaceutical dosage forms. The proposed method can be useful in quality control of bulk manufacturing and pharmaceutical dosage forms.

Keywords : Emtricitabine, Tenofovir alafenamide, HPLC, Development, Validation.

1. Introduction

Tenofovir alafenamide (TA) (Fig.1) is chemically isopropyl(2S)-2-[[[(1R)-2-(6-aminopurin-9-yl)-1-methyl-ethhoxy]methyl-phenoxy-phoshoryl]amino]propanoate. TA is a nucleotide reverse transcriptase inhibitor and a prodrug of tenofovir. It is used in the treatment of HIV infection and chronic hepatitis B. It is closely related to the commonly used reverse-transcriptase inhibitor tenofovir disproxil, TA has greater antiviral activity and better distribution into lymphoid tissues than that tenofovir disproxil.

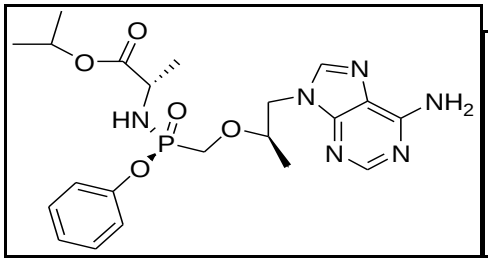

Fig.1: Structure of TA

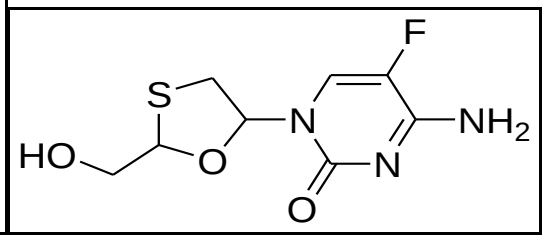

Fig.2: Structure of EMT

Emtricitabine (EMT) (Fig.2) is chemically 4-amino-5-fluoro-1-[(2R,5S)-2 (hydroxyl -methyl)-1,3-oxathiolan-5-yl]-1,2-dihydro- pyrimidin-2-one. EMT is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. EMT is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA.

TA and EMT in combination are available in tablet dosage forms containing 25 mg TA and 200 mg EMT. Literature survey shows that five UV spectroscopic[1,2,3,4,5] and five HPLC[6,7,8,9,10]analytical methods are reported for the estimation of EMT. For TA one method ofLC [11]and one spectroscopic method [12] reported in its bulk form. No method is reported for the simultaneous estimation of TA and EMT in bulk and pharmaceutical dosage forms. The purpose of this work was to develop a simple, basic, rapid and economic simultaneous RP-HPLC method for the determination of TA and EMT in bulk and pharmaceutical dosage forms so as to provide better scope for further research on the drugs. The proposed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelinesQ2(R1)[14].

2. Materials and Methods

2.1 Instrumentation

To develop a high pressure liquid chromatographic method for simultaneous estimation of EMT and TA by using Younglin (S.K) Gradient System HPLC. C_{18} column (250 x 4.6 mm) was used. The instrument is equipped with an UV 730 D detector. A 20 µLr heodyne injector port was used for injecting the samples.

2.2 Chemicals and solvents

The working standard of EMT and TA were obtained as gift samples from Mylan Pharmaceutical Ltd, Hyderabad, India. The tablet (Descovy) market formulation was procured from local market. HPLC grade water was purchased from Qualigens Ltd,Mumbai,India. Methanol (HPLC Grade) was obtained from E.Merck (India) Ltd, Mumbai, India.

2.3 Chromatographic conditions

Column: C18 column (250 x 4.6 mm)
Mobile phase: Methanol: Distill water
in proportion of 60:40 v/v (pH-3)
Detector: 260 nm
Injection volume: 20 µl
Flow rate: 1 ml/min
Temperature: Ambient
Run time: 10 min
Diluents: Mobile Phase

2.4 Selection of Mobile Phase

Standard solution of EMT and TA were injected into the HPLC system and run in different solvent systems. Mixture of different solvents were tried in order to determine optimum chromatographic conditions for effective separation of EMT and TA. After several permutation and combination, it was found that mixture of methanol: water gives satisfactory results as compared to other mobile phases. Finally, the optimal composition of the mobile phase methanol: water in the ratio of 60:40(v/v) (pH-3) was selected, as it gave high resolution of EMT and TA with minimal tailing.

2.5 Preparation Mobile Phase

Mobile phase was prepared by mixing 60ml of methanol with 40ml of distilled water.The pH of mobile was adjusted by 0.05% ortho phosphoric acid (pH-3).This mobile phase was filtered through 0.45µ membrane filter and then it was ultrasonicated for 30 minutes.

2.6 Preparation of standard stock solution

About 10 mg of TA and 80 mg of EMT were accurately weighed & transferred to 10 ml volumetric flasks. Both the drugs were dissolved in 5 ml of mobile phase with shaking and then volume was made up to the mark with the mobile phase to get 1000µg/ml of TA and 8000µg/ml of EMT of standard stock solution of each drug. Then it was ultrasonicated for 10 minutes and filtered through 0.20µ membrane filter.

2.7 Selection of analytical wavelength

By appropriate dilutions of standard stock solution of TA and EMT were scanned separately on Jasco UV-spectrophotometer V-630 in spectrum mode between the wavelength range of 400 nm to 200 nm. The isobastic point was found to be 260 nm. Overlain spectrum of TA and EMT is shown in Fig no.3

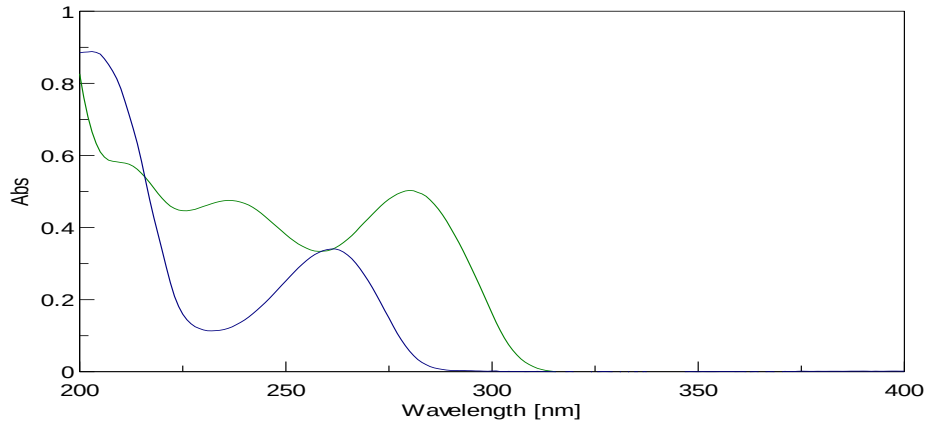

Fig. 3: Overlain spectrum of TA (261nm) and EMT (280nm)

2.8 Optimized Method:

- **Mobile phase:** Methanol: Water 60:40(v/v) (pH-3).
- ***λ* max:**260nm
- **Flow rate:**1ml/min
- **Retention Time:** 3.10& 7.38 min for EMT and TA

2.9 System suitability parameters

The System suitability parameters were studied and the results are summarized in Table no.1

Table 1: System suitability parameters

Sr.No.	Parameters	TA	EMT
1.	Retention time (min)	7.38	3.10
2.	Resolution (R)	12.2381	
3.	No. of theoretical plate (N)	6439.3	2997.5
4.	Tailing factor	1.2500	1.5714

2.10 Study of beers-Lambert's law

Preparation of standard calibration curves and selection of analytical concentration ranges:

From standard stock of 1000µg/ml of TA and 8000µg/ml of EMT appropriate aliquots were transferred to a series of 10 ml volumetric flasks. The volume was made up to mark with mobile phase to get set of solutions having concentration range 5-30µg/ml for TA and 40-240µg/ml for EMT. The diluted solutions were filtered through 0.2µ membrane filter. The filtrate (20µl) was injected into the column and chromatographed using optimized chromatographic conditions. The corresponding chromatograms were recorded and area of each peaks for TA and EMT were measured at 260 nm. Each sample solution was chromatographed in five time

and mean peak area for TA and EMT was calculated. Standard Calibration Graph for TA and EMT are given in Fig no.4 and Fig no.5.

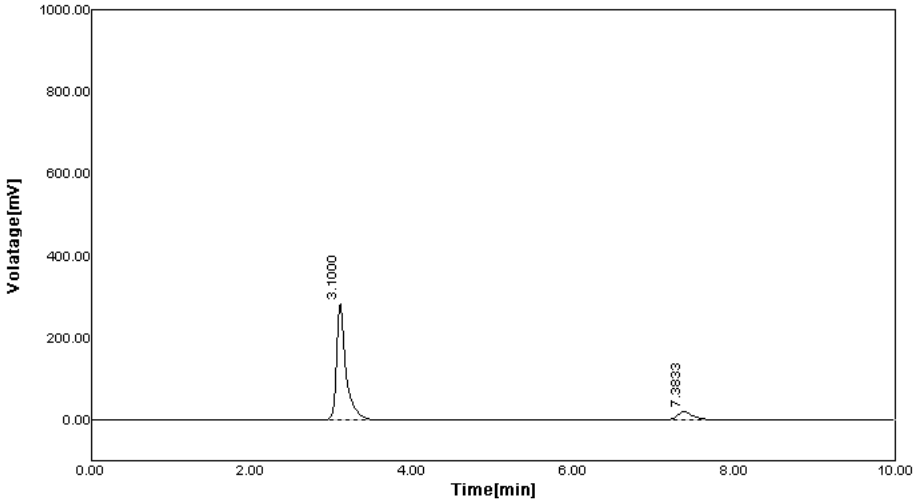

Fig.4: Chromatogram of TA and EMT

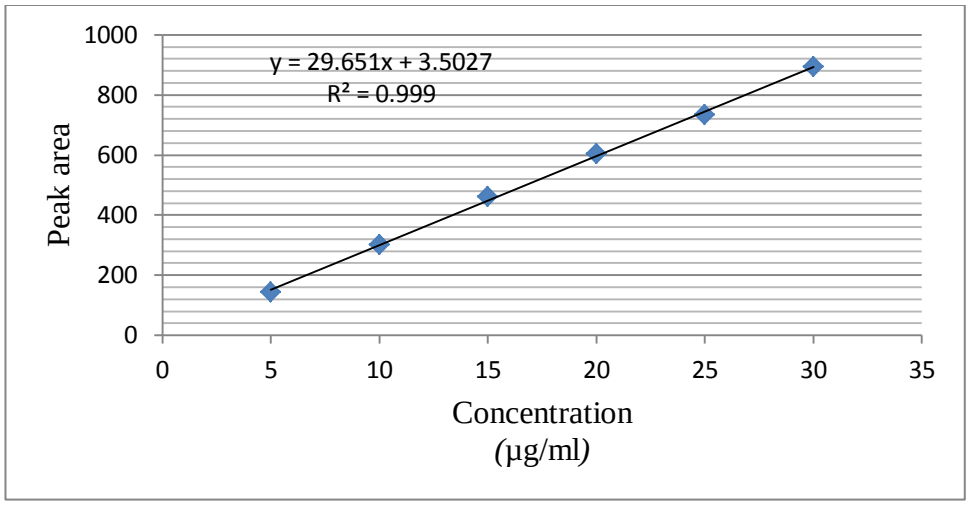

Fig. 5: Calibration curve of Tenofoviralafenamide by HPLC

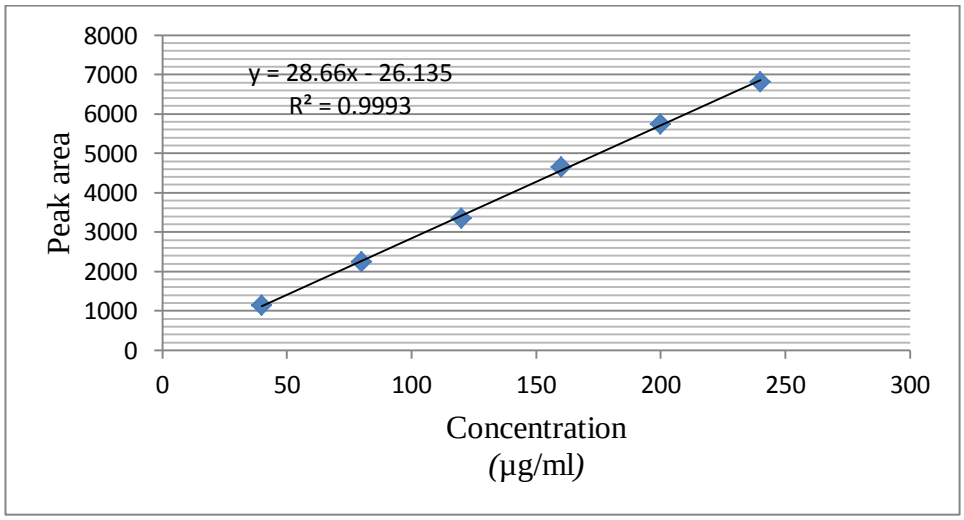

Fig. 6: Calibration curve of Emtricitabine by HPLC

3. Analysis of marketed formulation

For the estimation of drugs in the commercial formulations, twenty tablets containing 25mg of TA and 200mg of EMT were weighed and average weight was calculated. The tablets were crushed and powdered in glass mortar. For the analysis of drugs, quantity of powder equivalent to 10mg was transferred to a 10ml volumetric flask containing 5ml of mobile phase and then ultrasonicated for 20 min. Finally the volume was made up to the mark with mobile phase. The solution was filtered through Whatman filter paper no.42.This solution was further diluted with mobile phase to obtain mixed sample solution containing 25µg/ml of TA and 200µg/ml of EMT. The solution was filtered through 0.20 µ membrane filter. A 20 µl of sample solution was injected to into sample injector for six times under chromatographic condition as described above.Areas of each peak were measuredat260nm.The amount of each drug present in the sample was determined from peak area of TA and EMT present in the pure mixture respectively. The results are given in the Table no. 3 and Table no. 4.

Table 2: standard calibration curves

TA		EMT	
Conc. (µg/ml)	**Mean peak area**	**Conc. (µg/ml)**	**Mean peak area**
5	142.84	40	1131.57
10	301.54	80	2243.42
15	459.71	120	3354.03
20	604.18	160	4654.18
25	733.58	200	5730.46
30	892.50	240	6804.21

Table 3: HPLC Assay of TA and EMT (n=6)

Amt.taken (µg/ml)		Peak Area		Amt. found (µg/ml)		% of drug found	
TA	**EMT**	**TA**	**EMT**	**TA**	**EMT**	**TA**	**EMT**
25	**200**	735.57	5735.68	24.69	199.21	98.76	99.61
25	**200**	733.87	5728.67	24.63	198.97	98.52	99.49
25	**200**	736.47	5733.12	24.72	199.12	98.88	99.56
25	**200**	735.12	5736.94	24.67	199.26	98.68	99.63
25	**200**	737.65	5727.21	24.76	198.92	99.04	99.46
25	**200**	733.25	5729.11	24.61	198.98	98.44	99.49

Table 4: Statistical evaluation of marketed formulation

Drug	%Mean*	S.D.	%RSD
TA	735.32	1.63	0.22
EMT	5731.79	4.03	0.07

**Average of six determination*

Table 5:Linear regression data for calibration curves

Parameters	TA	EMT
Linearity range	05-30 µg/ml	40-240 µg/ml
r^2	0.999	0.999
Slope	29.65	28.66
Intercept	3.502	26.13

r^2: Correlation coefficient; S.E.: Standard error

Validation

The developed method was validated as per ICH guidelines.

4.1 Linearity and Range:

The linearity of measurement was evaluated by analyzing different concentrations of the standard solutions of TA and EMT. Beer's law was obeyed in the concentration range 5-30µg/ml and 40-240 µg/ml for TA and EMT respectively.

4.2 Precision

4.2.1 Repeatability

To check the degree of repeatability of the method, six samples of the marketed formulation were analyzed. The results of the Repeatability are given in Table no. 6.

Table 6: Result of repeatability

Drug	Conc. (µg/ml)	Area mean*	Amount found*	% Amount found*	S.D.*	%RSD*
TA	10	295.37	9.84	98.40	3.26	1.10
EMT	80	2323.96	80.17	100.21	1.46	0.06

**Average of six determination*

Table 7: Results of Intra-day and Inter-day precision studies

Parameter	Drug	Conc. (µg/ml)	Area mean*	Amount found*	% Amount found*	S.D.*	%RSD*
Interday	**TA**	5	149.25	4.91	98.20	1.48	0.99
		15	445.64	14.91	99.40	3.58	0.80
		25	733.10	24.60	98.40	4.04	0.55
	EMT	40	1185.59	40.83	101.08	4.17	0.35
		120	3483.57	120.63	100.53	5.92	0.17
		200	5771.27	200.44	100.22	79.81	1.38
Interday	**TA**	5	150.03	4.94	98.80	2.22	1.48
		15	447.64	14.97	99.80	1.93	0.43
		25	734.33	24.64	98.56	4.17	0.57
	EMT	40	1169.99	39.91	99.78	1.69	0.14
		120	3471.64	120.22	100.18	1.56	0.04
		200	5742.26	199.44	99.20	75.35	1.31

**Average of three determination*

4.2.2 Intermediate precision

The Intra and Inter-day precision was determined by analysis of the marketed formulation on the same day at different time intervals and on different days respectively. The results are given in Table no. 7.

Low %RSD values for intra and inter day confirmed that the method is precise.

4.3 Accuracy

To check the accuracy of the proposed method, recovery studies were carried out according to ICH guidelines by applying the standard addition method to known amount, of TA and EMT corresponding to 80,100 and 120%.Analysis was performed as per the procedure given under the tablet analysis. The recovery studies were performed three times at each level. The results of the recovery studies and its statistical evaluation are summarized in Table no.8 and Table no.9.

Table 8: Results of recovery studies

Recovery Level (%)	Drug	Conc. of drug(µg/ml)		Total conc. of drug (µg/ml)	Total amt. Recovered (µg/ml)	% Recovery*
		Drug Taken	Std drug added			
80	**TA**	5	4	9	9.04	101.02
100		5	5	10	9.99	99.43
120		5	6	11	11	100
80	**EMT**	40	32	72	72.31	100.95
100		40	40	80	80.10	100.25
120		40	48	88	87.89	101.58

**Average of three determination*

Table 9: Statistical evaluation of recovery studies

Level of recovery (%)	Drug	% recovery*	S.D.	%RSD
80	**TA**	101.02	1.09	1.08
100		99.43	0.89	0.90
120		100	1.15	1.13
80	**EMT**	100.95	1.09	1.08
100		100.25	0.46	0.46
120		101.58	0.49	0.48

**Average of three determination*

The recovery was found to be in the range of 99.43-101.02 % for TA, 100.25-101.58 % for EMT.

4.4 Robustness

The robustness of the method was studied, during development, by small but deliberate variations in flow rate, percentage of methanol in the mobile phase and wavelength. Each factor selected to examine were changed at three levels (-1, 0, 1)One factor at the time was changed to estimate the effect and to study the effect on the retention time of the drugs.The results are given in Table no.10.

Table 10: Robustness evaluation of TA and EMT

Sr. No.	Factor	Level	Retention time of EMT	Retention time of TA
1	**Flow Rate (ml/min)**			
	0.9	-1	2.93	7.25
	1	0	3.10	7.38
	1.1	+1	2.83	6.48
	Mean± S.D. (n=6)		2.95±0.13	7.03±0.48
2	**Mobile phase volume (v/v)**			
	59:41	-1	3.10	7.38
	60:40	0	3.10	7.38
	61:39	+1	3	7.27
	Mean± S.D. (n=6)		3.06±0.05	7.34±0.06
3	**Wavelength**			
	259	-1	3.10	7.18
	260	0	3.10	7.38
	261	+1	3.12	7.35
	Mean± S.D. (n=6)		3.10±0.01	7.30±0.10

Variation in flow rate, percentage of methanol in the mobile phase and wavelength did not affect the results. Rt and tailing factors of both the drugs at different levels of variations were similar. Hence, the method was found to be robust.

4.5 Limit of Detection (LOD)

` The limit of detection (LOD) is the smallest concentration that can be detected but not necessarily quantified as an exact value. LOD is calculated from the formula;

$LOD = 3.3\sigma / S$

Where, σ = standard deviation of the response, S = slope of calibration curve.

4.6 Limit of Quantitation (LOQ)

The limit of quantitation is the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy. LOQ is calculated from formula.

$LOQ = 10\sigma / S$

Where, σ = standard deviation of the response, S = slope of calibration curve

LOD, LOQ are shown in the Table no. 11.

Table 11:LOD and LOQ of TA and EMT

Parameter	TA	EMT
LOD(µg/ml)	0.3461	7.5153
LOQ(µg/ml)	1.0489	22.7739

Conclusion

From all results it was concluded that the developed RP - HPLC method for the simultaneous estimation of Tenofovir alafenamide and Emtricitabine in bulk and pharmaceutical dosage form was accurate, precise, linear, robust, simple and rapid. Percentage recovery shows that the method was free from interference of excipients used in the formulation.

Conflict of Interests

Declared None

Acknowledgement

Authors are grateful to Prof. M. N. Navale, President Sinhgad Technical Education Society for his continuous support and Mylan Laboratories Limited, Hyderabad, India for bulk drug gift sample.

Reference

1. Nagaraju P.T.,Channabasavaraj K.P., Shantha Kumar P. T., Development and Validation of Spectrophotometric Method for Estimation of Emtricitabine in tablet dosage form, International Journal of Chem. Tech Research ,Jan-Mar 2011,3(1), 23-28.
2. Sri K.V., Madhuri M., Jain G.V., Multivariate UV-visible spectrophotometric method for the estimation of Emtricitabine in bulk form, International Journal of Pharmaceutical Development & Technology,2015, 5(1)89-93.
3. Narendra A., Deepika D. and Annapurna M., Determination of Emtricitabine by Derivative Spectrophotometric Method in Pharmaceutical Dosage Forms,Chemical Science Transactions, 2013, 2(3), 978-982.

4. Kumar P, Mittan D.S., Kumar N., Development of new UV spectroscopic method for the estimation of Emtricitabine in bulk and capsuls ,Journal of Harmonized Research in Pharmacy, 2012,1(1), 44-47.
5. Srujani CH.,Sowmya D.K., Varanasi S N Murthy, A.Prameela Rani development and validation of Stability Indicating UV- spectrophotometric method for the estimation of Emtricitabine in bulk and its pharmaceutical dosage form ,An International Journal of Advances in Pharmaceutical Sciences , Jan-Feb 2016,7(1),3034-3039.
6. Sujatha K., Sheela Rani T., AnandBabu K. , Kavitha K. ,Chitra K., RP-HPLC Method Development and Validation of Emtricitabine in synthetic mixture, World Journal of Pharmaceutical Research, June 2014,3(6), 499-505.
7. Hamarapurkar P.D., and ParateAbhijeet N., HPLC Method for the Determination of Emtricitabine and Related Degradation Substances,Journal of Chromatographic Science,Jan2012, 51, 419–424.
8. Kumar V. Kiran and Raju N. Appala, Estimation of Emtricitabine in Tablet Dosage Form by RP-HPLC,Asian Journal of Chemistry ,2009, 21 (8) 5979-5983.
9. GojeArjun, Sathis Kumar. D,Yogeswaran. P, Sathyabrata Jena, David Banji, Rao KNV, A simple HPLC method for quantitation of Emtricitabine in capsule dosage form,Scholars Research Library , 2010, 2(2),281-285.
10. Seshachalam U., Bodepudi H., KottapalliB.Chandrasekhar, Development and validation of a stability-indicating liquid chromatographic method for determination of emtricitabine and related impurities in drug substance,Journal of separation science, 2007, 30, 999 – 1004 .
11. Mingxing Hu, Qi Wang, XiuyingMa,Chao Yang, HongbaoSun,Jie Liu, Yiwen Zhang, Yongmei Xi ,A Rapid and Sensitive LC Method for Determination of Diastereomeric Purity of Tenofovir Alafenamide, Chromatographia ,Oct 2014, 77(19) ,1399–1403.
12. Kumar P, Jacob J, Ajina K, Jazeela, Fathima, Spectroscopic Estimation of TenofovirAlafenamide, an antiretroviral drug, Research Journal Pharm. and Tech. 2016, 9(5),538-540
13. Beckett A. H. and Stenlake J. B., Practical Pharmaceutical Chemistry.4th edition, New Delhi: CBS Publishers and Distributors 2002,2, 275-288.
14. ICH Harmonized Tripartite Guideline Validation of Analytical Procedures: Text and Methodology, Q2(R1) November 2005, 1-17.
15. Sethi P. D., High Performance Liquid Chromatography, Quantitative Analysis of Pharmaceutical Formulations, 1st edition, CBS Publishers and Distributors, New Delhi 2001,3-11, 116-120.

*****