Analytical Method Development and Validation of Stability Indicating RP-HPLC Method for The Simultaneous Estimation of Dolutegravir, Emtricitabine and Tenofovir Disproxil Fumarate

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Abstracts: A simple, Accurate, precise method was developed for the simultaneous estimation of the Dolutegravir (DUA), Emtricitabine(ECB) and Tenofovir Disoproxil fumarate (TDF) tablet dosage form. Chromatogram was run through Std Kinetex Biphenyl, 250 x 4.6 mm, 5µm. Mobile phase-A containing pH-3.0 Ammonium acetate Buffer & Mobile phase -B containing Degassed mixture of Acetonitrile & Methanol taken in the ratio of 50:50 was pumped through column at a flow rate of 1 mL/min. Diluent used in this method was 0.1% Orthophosphoric acid buffer & Methanol in the ratio of 50:50%v/v. Temperature was maintained at 40°C. Optimized wavelength selected was 260 nm. Retention time of DUA, ECB and TDF were found to be 14.6min, 3.98min and 12.60min. %RSD of the DUA, ECB and TDF were found to be 0.35, 0.47 & 0.40 respectively. %Recovery was obtained as 98.9%, 99.6% and 99.3% for DUA, ECB and TDF respectively. Both the runtime and retention times were decreased, so this method developed was simple and economical this can be adopted in regular quality control in pharma industries.

Keywords: DUA, ECB and TDF, RP-HPLC, Decreasing, % Recovery, Buffer.

1. INTRODUCTION

The molecular formulae for Dolutegravir (DUA) is C20H19F2N3O5. This drug is available in the name as Tivicay. It is acting as an antiretroviral drug which is used, along other medication, for the treatment of disease named as HIV/AIDS.[1] This drug is also utilized, as part of post exposure prophylaxis, for the prevention of infections from HIV follows potential exposure.[2] It is taken with the help of mouth.[1]The drug named Emtricitabine is taken as combination by remaining antiretroviral agents to prevent and treat HIV-1 infection.[3][4]The studies revealed that by chronic infection of HIV, the viral replication should resume when the study subjects were taken out of therapy.[5] Tenofovir disoproxilis available as Viread along with others. It is medication utilized for treat hepatitis-B also for the prevention and to treat HIV/AIDS.[6] It is usually recommended to with other antiretrovirals.[6] A Cochrane review examined the use of Tenofovir for prevention of HIV before exposure and found that both Tenofovir individually as well as Tenofovir nor Emtricitabine association minimized the risk of contracting HIV to high risk patients.[7] RP-UPLC[8] method is developed to quantification of DUA sodium, ECB and TDF. For this authors are used Shimadzu NexeraX2 Model UPLC system with PDA detector and Shim-pack C18 column. Mobile phase is 0.1% Triethyl amine as 55v/v and Acetonitrile 45v/v. 1.0mL/min flow rate. 260 nm is wavelength. Concentration range is 5–400, 2–150 and 5–500 µg/mL for TDF, DUA sodium, and ECB, respectively. Run time is 5 min. LOQ are 1.9113, 4.8752, and 4.7654µg/mL. LOD are 0.6287, 0.1598, and 0.1568µg/mL. A stability-indicating RP-HPLC[9] technique was developed to EMT, TEN, DOL. Authors are used the column as Exterra C18 column (150×4.6mm, 5µm) and Methanol and Buffer as mobile phase in the proportion of 75:25 (v/v). 1mL/min is the elution time.

Wavelength is 265nm. Linear response is 500-1500µg/mL, 62.5-187.5µg/mL and 125-375µg/mL. The LOD values are 91.78µg/mL, 10.47µg/mL and 19.28µg/mL. The LOQ values are found and 278.11µg/mL, 31.74µg/mL and 58.42µg/mL. The assay is observed in between 99.11-100.84%. By using Exterra C18 column (150 × 4.6 mm, 5µm)[10]and Methanol and Buffer as75:25 (v/v), For accessing the stability of method capability, these are exposed to different environmental circumstances. The assay method with HPLC[11]is found as linear in strength as 15-150 µg/mL, 10-100 µg/mL and 30-300 µg/mL. Authors are used Phenomenex® C8 column (250 mm × 4.6 mm, 5µm). The mobile phase is potassium dihydrogen phosphate, acetonitrile and methanol as 40:40:20 v/v. The 1 mL/min is flow rate. 262 nm is wavelength. Antiretroviral combination [12] therapy regimens comprising of a nucleoside reverse transcriptase inhibitor (Emtricitabine, EMC), integrase inhibitor (Dolutegravir, DTG), and nucleotide reverse transcriptase inhibitor (Tenofovir Alafenamide, TAF) are frequently prescribed for HIV patients. This process is developed by using Qualisil-5 BDS C18 column (250mm × 4mm, 5µm). The mobile phase is acetonitrile 43v/v: orthophosphoric acid 57v/v. Flow rate is 1.2 mL/min using UV detection is 271nm. Injection volume is 20µL. The retention times are 8.321mins, 2.210mins, and 4.089 mins. Different authors [13,14,15] are determined the different drugs by using HPLC and Rp-HPLC methods, concluded that their methods are simpler and more accurate when compared with other methods as per their literature. By considering all these methods authors are adopted this simultaneous method for DUA, ECB and TDF.

2. MATERIALS & METHODS

The authors used the chromatographic separation by using Waters HPLC UV-Detector and Empower3 software. ODS C18 (4.6 x 250mm, 5 □m) column as stationary phase. This separation technique is performed by using cooling centrifuge C24 REMI at 5000 rpm, ultrasonic cleaner, Shimadzu analytical balance, vacuum microfiltration unit using 0.45µm nylon membrane filter. Acetonitrile, Ammonium Acetate, O-Phosphoric acid, Methanol, Water, Nitric acid, Formic Acid and water of HPLC grade from Merck. For this analysis, authors were used the reference samples Emtricitabine as 99.5%, Dolutegravir Sodium as 99.6% and Tenofovir Disoproxil as 98.2%.

2.1. Method Development

Method development for the estimation of DUA, ECB and TDF tablet dosage form was initiated based on the method development guidelines and literature review. Several trails were conducted by varying the chromatographic parameters for optimization of method. Separation of analytes was achieved by mobile phase containing acetonitrile, methanol, and water at a ratio of 60:10:30 (v/v/v). These are mixed, filtered by using vacuum filter and sonicated for 10min. delivered at a flow rate of 1.0mL/min through column Kinetex Biphenyl 250*4.6mm, 5µm or equivalent kept at 30 ºC. The volume of injection is 10 µL and runtime is finalized as 10 min. The eluents were observed at 260 nm. Buffer pH 3.0 along with 0.1% Orthophosphoricacid and Acetonitrile (80:20) was finalized. Based on peak parameters like resolution power, retention time, good peak shape, peak tailing, and no blank interference. Mobile phase A: pH3.0 Ammonium acetate buffer and Mobile phase B: ACN: Methanol as 70v/v: 30v/v. With the help of these solutions sharp peaks were appeared with good resolution so this was satisfactory and this mobile phase was finalized. For this analysis authors are prepared dilute formic acid solution, Buffer-1, Buffer-2, Mobile Phase-A, Mobile Phase-B, and Diluents. Along with these solutions authors prepared DUA standard stock solution, ECB standard solution and TDF standard stock solution and sample solution for further analysis.

3. RESULTS AND DISCUSSION

3.1. System Suitability

Five sample solutions were prepared for ECB, TDF and DUA. The solutions were injected into the HPLC system as per test procedure. The Resolution, theoretical plates, tailing factor and %RSD were calculated. USP plate count was determined. The solutions were injected six times and the parameters like peak tailing and resolution were observed. Evaluation of System suitability parameters were performed by considering the column efficiency as determined for the ECB, TDF and DUA peaks from standard solution is not less than 5000 theoretical plates and the tailing factor for the same peaks is not more than 2.0. The % RSD of the peak areas of ECB, TDF and DUA

obtained from five replicate injections of standard solution is not more than 2.0. The retention time for ECB, TDF and DUA peak is about 3.91min., 12.1min. and 14.37 min. respectively.

The system suitability parameters were determined by preparing standard solutions. The % RSD for the area of six standard injections results should not be more than 2%. The results are tabulated in the table 1.

Table1: System Suitability data for ECB, TDF and DUA

S.No	Sample Name	Name	RT	Area	USP Plate Count	USP Tailing
Mean	Std.,	ECB		2073851	89766	1.1
%RSD			0.0	0.2		
Mean	Std.	TDF	12.53	2991409	296504	1.3
%RSD			0.0	0.2		
Mean	Std.	DUA	14.53	1417061	291452	1.1
%RSD			0.0	0.3		

3.1. Specificity

Checking of the interference in the optimized method. Interfering peaks in blank and placebo at retention times of these drugs in this method were not identified. So, this method was said to be specific.

3.2. Precision

Six sample solutions were prepared. The chromatograms were observed and shown in Fig.1. The % RSD and % Assay of ECB**,** TDF and DUA are found to be 0.4, 0.4, 0.4 and 100.5, 99.2 and 99.6 respectively. The % RSD should be NMT 2.0% and Average % Assay should be between 95-105% of labeled amount. The obtained values are tabulated in table 2.

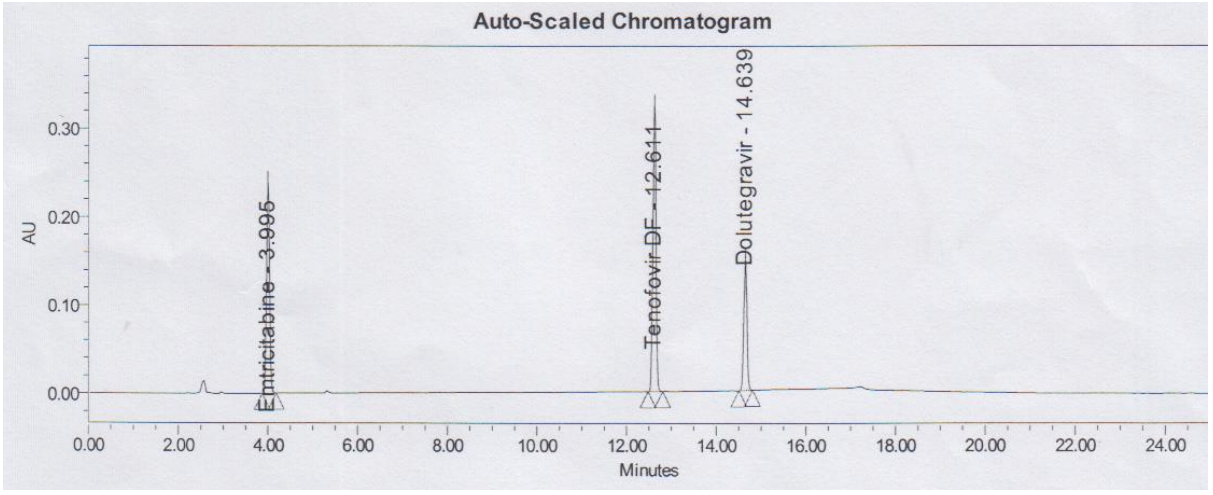

Figure 1 Chromatogram of precision

Table: 2 System Precision and method precision for ECB, TDF and DUA

S.No	Sample Name	ECB	TDF	DUA	ECB	TDF	DUA
		Method Precision Data			System Precision Data		
1	Precision sample-1	2072946	2991518	1418517	100.5	99.2	99.9
2	Precision sample-2	2087056	3014857	1429090	101.2	99.9	100.6
3	Precision sample-3	2076215	2996687	1421532	100.7	99.3	100.1
4	Precision sample-4	2068221	2987971	1418067	100.3	99.0	99.8
5	Precision sample-5	2067345	2988144	1415716	100.2	99.1	99.7
6	Precision sample-6	2065185	2984327	1414887	100.1	98.9	99.6
Mean		2072828	2993917	1419635	100.5	99.2	100
Std. Dev.		8045.183	11068.534	5188.569	0.4	0.4	0.4
%RSD		0.4	0.4	0.4			

3.3. Linearity

The chromatograms of Dolutegravir, Emtricitabine and Tenofovir DF were taken into consideration. The peak areas were observed. To analyze this parameter DUA, ECB and TDF Standard stock solution, Preparation of 25%, 50%, 75%, 100%, 125% and 150% solution (Level-1,2,3,4,5 and 6) were prepared. Correlation coefficient and % y-Intercept for DUA, ECB and TDF were 0.999949 and -4781.443999; 0.999823 and-27763.69635; 0.999831and-55379.8565 respectively. Correlation coefficient should be more than 0.9 and % y-Intercept should be not more than 5%. The values obtained were represented in the table 3.

Table3: Linearity data of DUA, ECB and TDF

S.No	Linearity level	Conc.(ppm)	Response	Conc.(ppm)	Response	Conc.(ppm)	Response
		DUA		ECB		TDF	
1	Level-1	20.32	707020	80.78	1039374	121.04	1509464
2	Level -2	38.09	1334520	121.17	1564004	181.56	2267807
3	Level -3	40.63	1426371	161.56	2067808	242.08	2992870
4	Level -4	50.79	1780972	193.87	2509460	290.50	3624567
5	Level -5	60.95	2124325	242.34	3082741	363.12	4451552
Correlation coefficient			0.999949		0.999823		0.999831
%y-intercept			-4781.443999		-27763.69635		-55379.8565

3.4. Accuracy

To determine the accuracy of the method recovery was performed by standard addition method. To pre-analyzed sampled, known number of standards. For this parameter authors prepared Standard stock solution, 50%, 100% and 150% Spiked Solution. ECB, TDF and DUA were spiked in different concentrations. The recoveries of ECB, TDF and DUA were at three levels 50%, 100% and 150%. Accuracy was found to be 99.6%. The % recovery of ECB, TDF and DUA at each spiked level should be not less than 98% and not more than 102%. The % Recovery for each level should be between 98.0 to 102. The results are shown in table 4.

Table 4. Accuracy Data of DUA, ECB and TDF

S.No	Accuracy level	%Recovery	Mean recovery	Overall mean recovery
1	**DUA-**50%	99.4,98.4,98.7	98.8	**99.1**
2	**DUA-**100%	99.1,99.9,99.1	99.6	
3	**DUA-**150%	98.7,99.5,98.5	98.9	
4	**ECB-**50%	99.6,99.7,99.6	100.6	**100.3**
5	**ECB-**100%	100.6,99.9,100.5	99.9	
6	**ECB-**150%	99,100.5,99.4	100.5	
7	**TDF-**50%	99.3,99.9,98.8	99.3	**99.5**
8	**TDF-**100%	99.9,98.5,99.4	99.3	
9	**TDF-**150%	99.9,99.9,100.2	100	

3.5. Robustness

Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH guide lines. Robustness conditions like Flow minus (0.9 mL/min), Flow plus (1.1mL/min), mobile phase minus, mobile phase plus, temperature minus (25°c) and temperature plus (40°c) were maintained, and samples were injected in duplicate manner. System suitability parameters were not much effected, and all the parameters were passed. %RSD was within the limit. To prove the Robustness of the method, different parameters were also verified. 1.2mL Flow, 10°c Sample cooler temperature, Mobile phase. By changing the flow, Mobile phase and temperature were no recognized change in the results and are within range as per ICH Guidelines. The % difference found for PVDF filter 0.0, 0.3 and 0.0 & Nylon filters 0.1, 0.0, and 0.3 for DUA, ECB and TDF Respectively. % drug release results of centrifuged versus filtered samples should be not more than 2.0%. The values are shown in the table 5, 6 & 7.

Table 5. Robustness Data of DUA, ECB and TDF

S.No	Robustness Condition	ECB	TDF	DUA
1	Flow minus	0.1	0.0	0.2
2	Flow plus	0.0	0.1	0.1
3	Mobile phase minus	0.6	0.3	0.9
4	Mobile phase plus	0.2	0.3	0.4
5	Temperature minus	0.1	0.3	0.1
6	Temperature plus	0.9	1.6	1.3

Table 6. % RSD for ECB, TDF and DUA

S.No	Sample Name	ECB	TDF	DUA
1	Sample (PVDF)	2073159	2990271	1413241
2	Sample (Nylon)	2078269	2991552	1417986
3	Sample (Centrifuged)	2074874	2995694	1421402
Mean		2073851	2991409	1417061
Std. Dev.		4864.55	7196.08	4008.69
%RSD		0.2	0.2	0.3

Table 7. Data for % Difference to ECB, TDF and DUA

%Assay	Centrifuged	PVDF	%Difference	Nylon	%Difference
Emtricitabine	99.9	99.9	0	100	-0.1
Tenofovir DF	98.8	98.5	0.3	98.8	0
Dolutegravir	102.6	102.6	0.0	102.9	-0.3

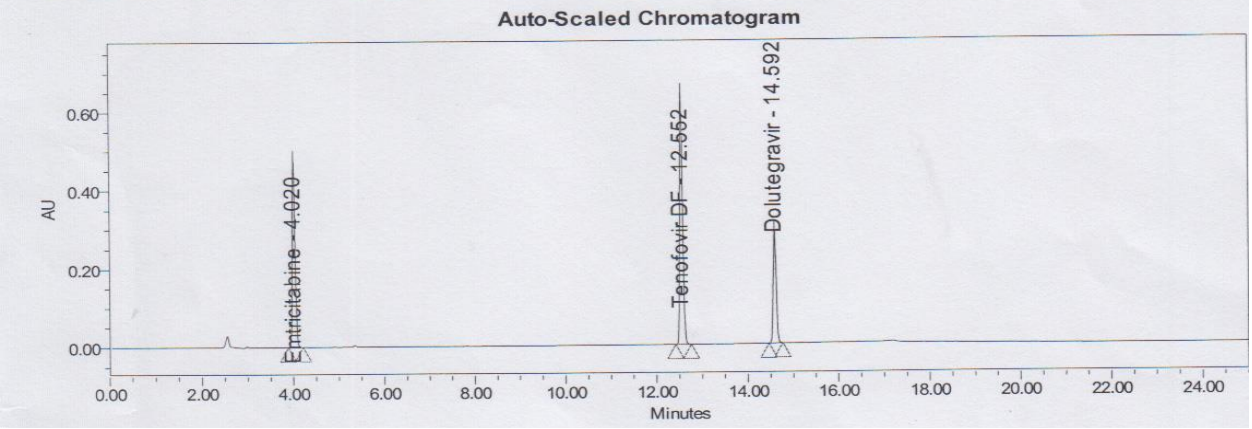

Figure 2: Chromatogram for ECB, TDF and DUA - Nylon

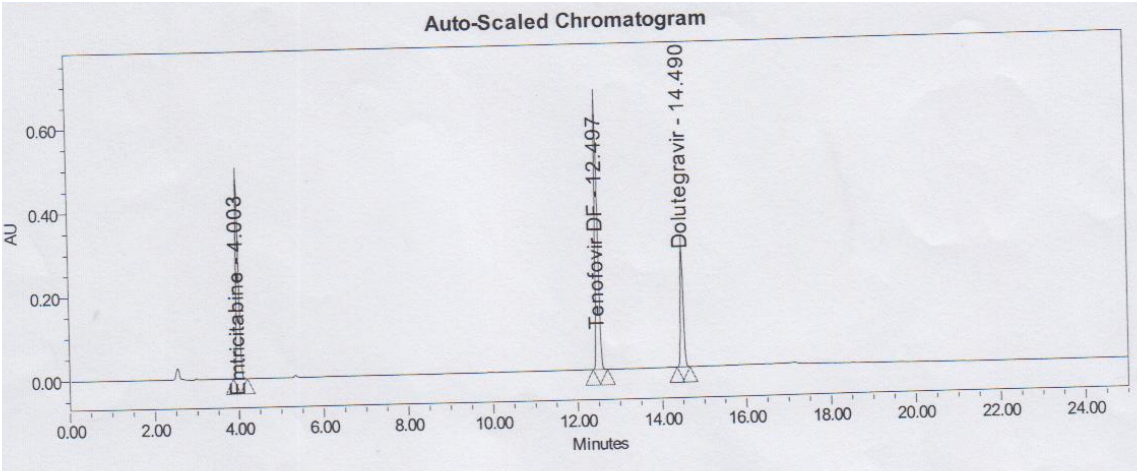

Figure 3: Chromatogram for ECB, TDF and DUA - PVDF

3.6. Degradation Studies

Different degradation studies which includes oxidation, acid degradation studies, alkali degradation studies, dry heat degradation studies, photo stability studies and neutral degradation studies were performed. From the results, it is concluded that purity angle is less than purity threshold in all the stress conditions. Purity angle should be less than purity threshold. Emtricitabine, Tenofovir Disoproxil Fumarate and Dolutegravir were eluted at 4.020 min, 12.552 min and 14.592 min respectively with good resolution. Plate count and Tailing factor was very satisfactory. Hence, method was optimized and to be validated. The obtained values are shown in table 8.

Table 8. Result for forced degradation studies of ECB, TDF and DUA

Sample Name	Condition	Purity angle	Purity Threshold
Emtricitabine	Control Sample	0.122	0.293
	0.1N Hydrochloric acid at 80^0C/15min	0.123	0.316
	0.1N Sodium hydroxide/ 5mins	0.124	0.303
	3% Hydrogen peroxide 80°C/60mins	0.146	0.302
Tenofovir DF	Control Sample	0.106	0.239
	0.1N Hydrochloric acid at 80^0C/15min	0.090	0.240
	0.1N Sodium hydroxide/5mins	0.088	0.235
	3% Hydrogen peroxide 80°C/60mins	0.122	0.243
Dolutegravir	Control Sample	0.086	0.213
	0.1N Hydrochloric acid at 80^0C/15min	0.016	0.209
	0.1N Sodium hydroxide/ 5mins	0.016	0.209
	3% Hydrogen peroxide 80°C/60mins	0.016	0.211

3.7. Solution Stability

Solution stability was performed by injecting standard and sample solution after 12Hrs and 24Hrs at 5°C. The solution stability results are shown in table 9.

Table 9. Solution stability data of ECB, TDF and DUA

S.No	RT	Area	USP Plate count	USP Tailing
ECB				
Mean	4.0	2074024	95415	1.0
%RSD	0.0	0.2	330698	0.9
TDF				
Mean	26.795	2967359	295156	1.3
%RSD	0.0	3.0	300678	1.1
DUA				
Mean	30.274	1353164	290252	1.1
%RSD	0.0	0.8	301598	0.9

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the DUA, ECB & TDF in tablet dosage form. Retention times for DUA, ECB & TDF were identified at lower Rt level. % RSD and % Recovery of the DUA, ECB & TDF were measured. LOD, LOQ values were calculated from regression equations of DUA, ECB&TDF. So, the method developed was simple and economical that can be adopted in regular Quality control analysis in pharma industries.

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Conflicts of Interest

There are no conflicts of interest among the authors.

REFERENCES

[1] British national formulary: BNF 69 (69 ed.). British Medical Association. 2015. p. 429. ISBN 9780857111562.

[2] "Dolutegravir Sodium Monograph for Professionals". Drugs.com. Retrieved 20 April 2019.

[3] "Emtriva- emtricitabine capsule Emtriva- emtricitabine solution". DailyMed. 14 December 2018. Archived from the original on 24 July 2020. Retrieved 24 July 2020.

[4] "Emtriva EPAR". European Medicines Agency. 17 September 2018. Archived from the original on 24 July 2020. Retrieved 24 July 2020.

[5] Oxenius A, Price DA, Günthard HF, Dawson SJ, Fagard C, Perrin L, "Stimulation of HIV-specific cellular immunity by structured treatment interruption fails to enhance viral control in chronic HIV infection". Proceedings of the National Academy of Sciences of the United States of America. 2002; 99 (21): 13747–52. Bibcode:2002PNAS...9913747O. doi:10.1073/pnas.202372199. PMC 129766. PMID 12370434.

[6] "Tenofovir Disoproxil Fumarate". The American Society of Health-System Pharmacists. Archived from the original on 30 November 2016. Retrieved 29 November 2016.

[7] Okwundu. CI, Uthman. OA, Okoromah. CA. "Antiretroviral pre-exposure prophylaxis (PrEP) for preventing HIV in high-risk individuals". The Cochrane Database of Systematic Reviews. 2012;7 (7):CD007189. doi:10.1002/14651858.CD007189.pub3.

[8] Annapurna, D. M. M. Simultaneous Estimation of Dolutegravir Sodium, Emtricitabine, and Tenofovir Disoproxil Fumarate by UPLC. AJP 2022, 16.

[9] Rathod SM, Patel NC, Patel PU. Simultaneous determination of Emtricitabine, Tenofovir alafenamide fumarate and Dolutegravir sodium by validated stability-indicating RP-HPLC-DAD method. Ann Pharm Fr. 2023;81(1):94-106. doi: 10.1016/j.pharma.2022.08.006. Epub 2022 Aug 28. PMID: 36037931.

[10] S.M. Rathod, N.C. Patel, P.U. Patel, Simultaneous determination of Emtricitabine, tenofovir alafenamide fumarate and Dolutegravir sodium by validated stability-indicating RP-HPLC-DAD method, Annales Pharmaceutiques Françaises, 2023; 81(1), 94-106, https://doi.org/10.1016/j.pharma.2022.08.006.

[11] Mehdi Rezaei, Ali Ramazani and Fahimeh Hokmabadi Simultaneous Estimation and Validation of Tenofovir Disoproxil Fumarate, Emtricitabine and Efavirenz by RP-HPLC Method in Combined tablet Dosage Form; 2019; 15(6), 2019: 561 – 567; DOI: 10.2174/1573412914666180208152021

[12] Prashant Ghode, D, Atul Sayare. S, Asawari Pachauri. D, Anil. Tankar, Saurabh Shinde. U, Dattatray Saindane. S, Vivekanand. Tembhurnikar, Shweta Ghode. P; RP-HPLC method development and validation for the simultaneous estimation of Dolutegravir, Emtricitabine, and Tenofovir alafenamide in tablet dosage form; Journal of medical pharmaceutical and allied sciences, 2022; 11(2), 4734 – 4740; DOI: 10.55522/jmpas.V11I2.2286

[13] Ch. V. Kameswara Rao, K. Ramakrishna, V. D. N. Kumar Abbaraju, Umamaheswara Rao Tadikamalla and Dunna Rahul; “Development Of Method And Validation For Assay In Irinotecan Hydrochloride Injection By Applying Stability Indicating Hplc Methodology By Hplc”; Rasayan Journal of Chemistry; 2022; 15(4): 2893-2900 DOI: http://doi.org/10.31788/RJC.2022.1547057

[14] Supriya. B S, and Avinash. Krishnegowda; Development And Quantification Of Horseradish Peroxidase Using Paraphenylenediamine And Alpha Naphthol: Applications In Medicinal Plant Sources; Rasayan J. Chem., 2022; 15(4), 2993-2998. http://doi.org/10.31788/RJC.2022.1548091

[15] P. Shelar, C. Nazareth, R. Khorjuwenkar, O. Pinto, G. Kamat and B. Naik; “A Novel Uv Spectrophotometric Method For Simultaneous Estimation Of Metoprolol Tartrate And Atorvastatin Calcium Based On Absorbance Correction Principle” Rasayan J. Chem., 2022; 15(4), 2822-2827() http://doi.org/10.31788/RJC.2022.1547065

DOI: https://doi.org/10.15379/ijmst.v10i3.3280