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Research Article

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METHOD DEVELOPMENT AND VALIDATION FOR MULTI-COMPONENT ANALYSIS OF LAMIVUDINE & TENOFOVIR DISOPROXIL FUMARATE IN BULK DRUG BY UV-VISIBLE SPECTROPHOTOMETER & RP-HPLC

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ABSTRACT
A novel, simple, precise and accurate method developed for the estimation of Lamivudine and tenofovir disoproxil fumarate (TDF) in bulk drug form has been established. Lamivudine and tenofovir are well known drugs and used in treatment of HIV- Ⅰ. The method was performed by using C18 column, ODS Hypersil column with UV detection at 262nm by using Acetonitrile and water in ratio 55:45. The retention time was found to be 2.8 and 6.8 min for Lamivudine and tenofovir disoproxil fumarate (TDF). The linearity was found in range of 6- 14µg/ml for Lamivudine and 10- 50µg/ml for Tenofovir disoproxil fumarate with flow rate 1ml/min. the method was validated for linearity, accuracy, precision and robustness as per ICH guidelines. This method is suitable for simultaneous analysis for both the nucleoside analog reverse- transcriptase inhibitors

INTRODUCTION

Tenofovir disoproxil fumarate (Fig-1) and Lamivudine (Fig-2) are widely used anti-retroviral drugs in the categories of NRTIs i.e. nucleotide analogues reverse transcriptase inhibitors [1-4]. These drugs are used for the prevention and clinical management of acquired immune deficiency syndrome (AIDS) with multiple complications [5-8].

Lamivudine is commonly called 3TC used in the treatment of HIV/AIDS and also treat chronic hepatitis B where the virus that causes complicated liver inflammation. It slow down and prevent damage to immune system and reduce the risk of developing AIDS related illnesses. Lamivudine help fight the virus and slow the ability to damage liver [9-12].

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Tenofovir is type of anti-HIV medicine called a nucleoside reverse transcriptase inhibitor (NRTI). It is always used in combination with other antiviral agents to treat patients with HIV. It is used in the form of pro- drug as Tenofovir Disoproxil Fumarate [13-15]. Tenofovir is not cure for HIV infection but decrease risk of spreading HIV disease to others. It also used to treat the certain type of liver infection called chronic hepatitis B infection [16-18]. The literature review revealed that there are several methods available for single component analysis for lamivudine and Tenofovir (TDF) [19-20].

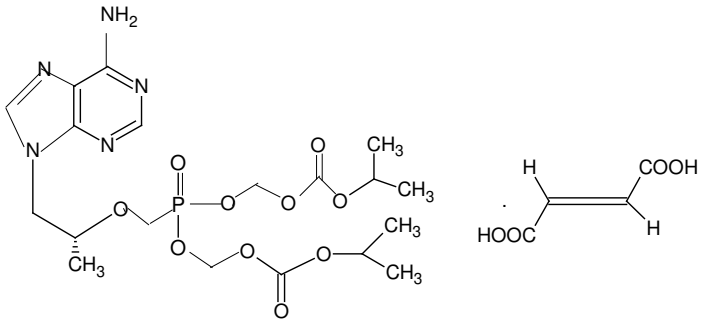

Figure 1: Tenofovir (TDF)

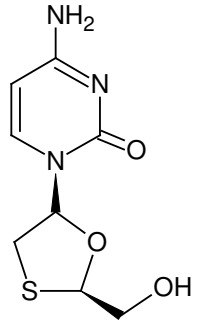

Figure 2: Lamivudine

MATERIAL AND METHOD

Instrument

The lambda max and iso-absorptive point were determined by UV –spectrophotometer using Lab India with UV win software. HPLC (Shimadzu) prominence LC 20 AD, manual sampler, software LC solution and detector (UV-visible), Column C-18, Thermo scientific octadecylsilane Hypersil (ODS), ultra-sonicater, vacuum filter, analytical balance.

Selection of wavelength

The selection of wavelength 10 µg/ml concentration of lamivudine and 10µg/ml concentration of tenofovir was prepared in 55:45 with ACN: water respectively. The result show iso-absorptive point that was observed at 262 nm (figure 3).

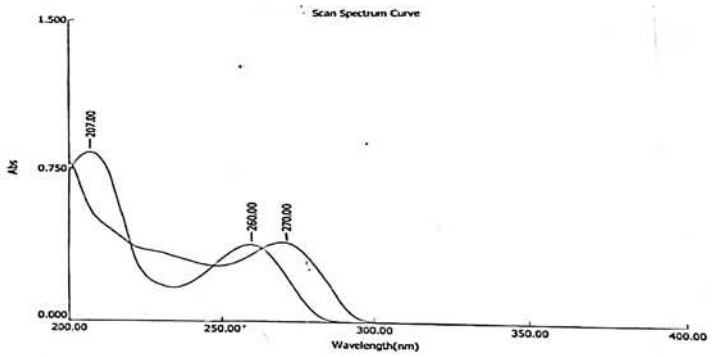

Figure 3. Overlain spectrum of lamivudine and TDF

Selection of chromatographic condition

The isocratic mode with mobile phase Acetonitrile and water in ratio 55:45 with flow rate 1ml/min. The resulting chromatograms were recorded and the chromatographic responses were measured.

Analytical Method Validation

A calibration curve was plotted with the concentration range 6-14µg/ml for lamivudine and 10-50µg/ml for tenofovir (TDF). The method was developed and validated as per ICH guidelines. The parameters were studied linearity, accuracy, precision (intraday and interday precision and repeatability) and robustness and the amount recovery, percentage recovery and mean recovery for the same was calculated.

Preparation of standard stock solution

Lamivudine standard stock solution

Standard lamivudine 100 mg was weighed and transferred to a 100 ml clean and dry volumetric flask and dissolved into the HPLC grade sample solution (ACN: Water in the ratio 55:45) then volume was made up to the mark with solution containing 1000µg/ml conc. Then 10 ml of solution was pipette out and transferred to 100 ml clean and dry volumetric flask, made up its volume with solvent to get 100 µg/ml conc. solutions.

TDF standard stock solution

Standard tenofovir (TDF) 100 mg was weighed and transferred to a 100 ml clean and dry volumetric flask and dissolved into the HPLC grade sample solution (ACN: Water in the ratio 55:45) then volume was made up to the mark with solution containing 1000 µg/ml conc. Then 10 ml of solution was pipette out and transferred to 100 ml clean and dry volumetric flask, made up its volume with solvent to get 100 µg/ml conc. solutions.

Chromatographic conditions

The mobile phase consisting of Acetonitrile: water (55:45) was used and absorbance was measured at 262 with the run time 15 min and the flow rate was set at 1.0 ml/min respectively.

Preparation of mobile phase

Mobile phase was prepared by mixing HPLC grade acetonitrile and water in ratio of 55: 45 respectively, and the chromatographic conditions were made for separation of the drugs at the wavelength of 262nm. Degassing is done before the use of mobile phase.

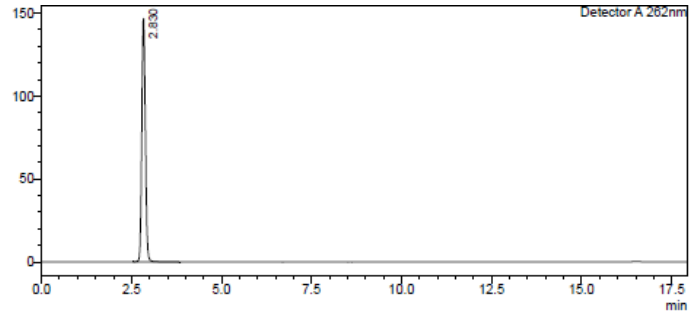

Figure 4: Chromatogram of lamivudine at wavelength 262nm

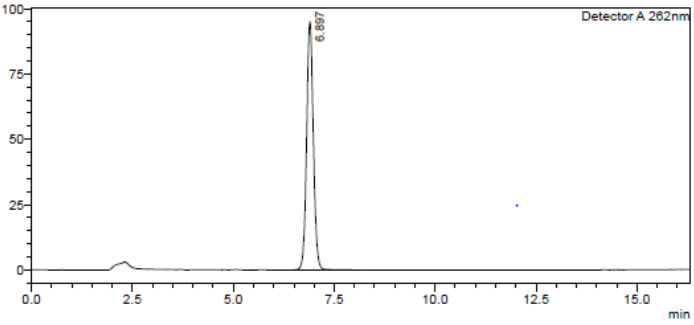

Figure 5: Chromatogram of tenofovir (TDF) at wavelength 262nm

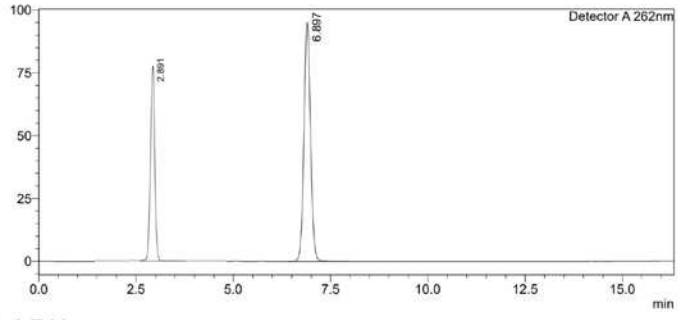

Figure 6: Chromatogram of both drugs at 262nm

Validation of the developed method

Linearity curve for lamivudine

Standard lamivudine stock solution the volume of 0.6, 0.8, 1, 1.2, 1.4ml was pipetted out from 100µg/ml and transferred to different 10 ml clean and dry volumetric flasks. The volume was made up to the mark having conc. of 6, 8, 10, 12, 14µg/ml respectively. The injection was prepared 20µg/ml and given with run time of 15 minutes. The linearity peaks was found to be within the limits. The results are shown in figure 7 and table 1.

Table: 1. Results of linearity curve of Lamivudine at wavelength 262 nm.

S. No	Conc. (µg/mL)	Area (µ volt sec.)
1	6	376003
2	8	651314
3	10	866679
4	12	1108256
5	14	1357247

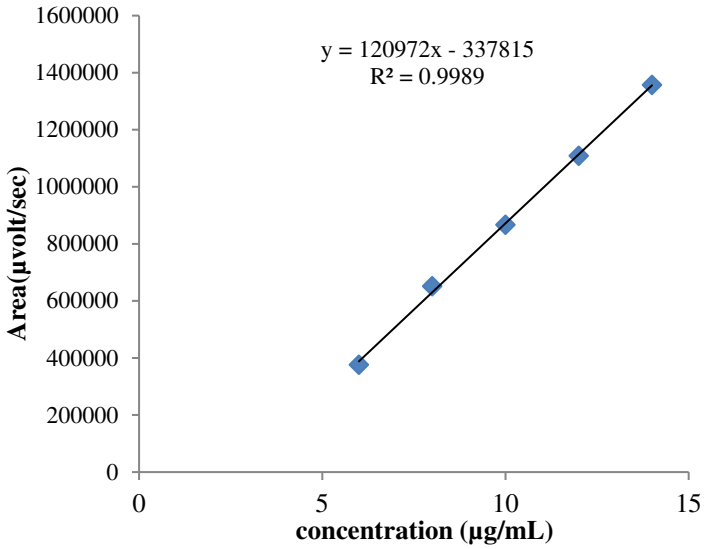

Figure 7: Linearity curve of lamivudine at 262nm

Linearity curve for tenofovir (TDF)

Standard tenofovir (TDF) stock solution the volume of 1, 2, 3, 4, 5, 6 ml was pipetted out from 100µg/ml and transferred to different 10ml clean and dry volumetric flasks. Then volume was made up to the mark having conc. of 10, 20, 30, 40, 50, 60µg/ml respectively. The injection was given 20µg/ml with run time of 15 minutes. The linearity peaks was found to be within the limits. The results are shown in figure 8 and table 2.

Accuracy

To study the accuracy, 3 determinants of conc. range of 8, 10, 12µg/ml for lamivudine and 20, 30, 40 µg/ml for tenofovir (TDF0 were prepared having 80%, 100%, and 120% of spiked level respectively. 3 replicates of above conc. were prepared and

responses were obtained. Percent recovery was calculated for obtained data and calculated according to ICH guidelines (Table 3 and 4).

Table 2. Result of linearity curve of tenofovir (TDF) at 262nm

S. No	Conc. (µg/mL)	Area (µ volt sec.)
1	10	427228
2	20	733996
3	30	1101386
4	40	1481526
5	50	1916508

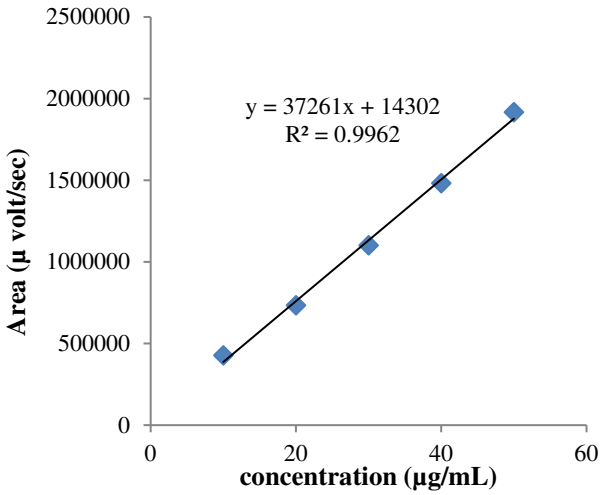

Figure 8. Linearity curve of tenofovir (TDF) at 262nm

Drug recovery

$$80\% = \frac{(\text{Mean of abs. of 180\% fortified sample} - \text{mean abs.of 80\% unfortified sample})}{\text{Mean abs.of fortified standard solution of 100\% test conc.}} \times 100$$

$$100\% = \frac{(\text{Mean abs.of 200\% fortified sample} - \text{Mean abs.of 100 unfortified sample})}{\text{Mean abs.of fortified standard solution of 100\% test conc.}} \times 100$$

$$120\% = \frac{(\text{Mean abs.of 220\% fortified sample} - \text{Mean abs.of 120\% unfortified sample})}{\text{Mean abs.of fortified standard solution of 120\% test conc.}} \times 100$$

Table 3: % Drug Recovery of lamivudine at wavelength 262nm

S. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/ml)	Area	Mean	Conc. (µg/ml)	Area	Mean	
1	**8**	651315 651313 651314	651314	**8+10**	1517973 1517971 1517970	1517971	**99.34**
2	**10**	866680 866678 866679	866679	**10+10**	1733398 1733400 1733396	1733398	**100.02**
3	**12**	1108257 1108255 1108254	1108257	**12+10**	1974929 1974927 1974927	1974927	**99.05**

Table 4: % Drug recovery of tenofovir (TDF) at wavelength 262nm

S. No.	Unfortified sample			Fortified sample			% Recovery
	Conc. (µg/ml)	Area	Mean	Conc. (µg/ml)	Area	Mean	
1	**20**	733996 733976 733996	733989	**20+30**	1835268 1835270 1835269	1835269	**99.06**
2	**30**	1101386 1101389 1101387	1101388	**30+30**	2202799 2202797 2202799	2202798	**100.0**
3	**40**	1481526 1481429 1481428	1481461	**40+30**	2582813 2582811 2582812	2582812	**99.76**

Precision

The precision was done for interday, intraday and repeatability.

Interday & intraday precision

Interday & intraday precision of conc. 8, 10, 12µg/ml was prepared and data was obtained for lamivudine.

Interday & intraday precision of conc. 20, 30, 40µg/ml was prepared and data was obtained for tenofovir (TDF). 3 replicates were prepared for 3 days.

The results of lamivudine and tenofovir were shown in table 5 to 7.

Table 5: Intraday precision of lamivudine and tenofovir(TDF) at 262nm

Drug	**Lamivudine**			**Tenofovir (TDF)**		
Conc.	**8 µg/ml**	**10 µg/ml**	**12 µg/ml**	**20µg/ml**	**30µg/ml**	**40µg/ml**
Area (µ volt sec.)	651314	866679	1108256	733996	1101386	1481526
	651294	866580	1108350	733954	1101285	1481429
	651229	866662	1108275	733889	1101324	1481494
Mean	651279	866640.3	1108294	733946.3	1101332	1481483
S D	44.440	52.937	49.702	53.910	50.934	49.426
% RSD	0.0068	0.0061	0.0044	0.007	0.004	0.003

Table 6: Interday precision of lamivudine at 262nm

Day	**Day 1**			**Day 2**			**Day3**		
Conc.	**8µg/ml**	**10µg/ml**	**12µg/ml**	**8µg/ml**	**10µg/ml**	**12µg/ml**	**8µg/ml**	**10µg/ml**	**12µg/ml**
Area (µ volt sec.)	651314	866679	1108256	651379	866679	1108256	651314	866679	1108256
	651294	866580	1108350	651314	866558	1108344	651226	866685	1108375
	651229	866662	1108275	651215	866679	1108298	651289	866589	1108226
Mean	651279	866640.3	1108294	651302.7	866638.7	1108299	651276.3	866651	1108286
S D	44.440	52.937	49.702	82.585	69.859	44.015	45.346	53.777	78.805
%RSD	0.0068	0.0061	0.0044	0.012	0.008	0.003	0.0069	0.0062	0.0071

Table 7: Interday precision of tenofovir (TDF) at 262nm

Day	**Day 1**			**Day 2**			**Day 3**		
Conc.	**20µg/ml**	**30µg/ml**	**40µg/ml**	**20µg/ml**	**30µg/ml**	**40µg/ml**	**20µg/ml**	**30µg/ml**	**40µg/ml**
Area (µ volt sec.)	733996	1101386	1481526	733987	1101287	1481437	733996	1101386	1481432
	733954	733954	1481429	733996	1101386	1481525	733887	1101476	1481526
	733889	733889	1481494	733999	1101296	1481516	733992	1101394	1481489
Mean	733946.3	733946.3	1481483	733994	1101323	1481493	733958.3	1101419	1481482
S D	53.910	53.910	49.426	6.244	54.744	48.418	61.80885	49.81298	47.35328
%RSD	0.007	0.007	0.003	0.0008	0.0049	0.0032	0.0084	0.0045	0.0031

Repeatability

For repeatability determination minimum of 6 determinants were prepared of 20µg/ml conc. and the chromatogram responses were obtained. The results of lamivudine and tenofovir (TDF) were shown in table 8

Table 8: Repeatability of lamivudine and TDF at 262nm

S. No	Area (µ volt sec.)	Area (µ volt sec.)
1	866699	1101386
2	866624	1101285
3	866612	1101324
4	866682	1101386
5	866645	1101476
6	866657	1101394
Mean	866645	1101332
S D	47.148	50.934
%RSD	0.005	0.0046

Robustness

This method was carried out by changing wavelength and flow rate of mobile phase. The results were shown in table 9 for change in mobile phase and table 10 for change in flow rate.

Table: 9 Robustness of lamivudine & tenofovir (TDF) at wavelength 262±2nm.

Wavelength	Difference	R_t of Lamivudine (min.)	R_t of Tenofovir (min.)
260	-2	2.769	6.883
262	0	2.858	6.881
264	+2	2.841	6.888

Change in flow rate of mobile phase

Table: 10 Robustness of lamivudine & tenofovir (TDF) at wavelength 262 nm.

Flow rate (mL/min.)	Difference	R_t of Lamivudine (min.)	R_t of Tenofovir (min.)
0.9	-0.1	2.752	6.780
1	0	2.858	6.880
1.1	+0.1	2.285	6.898

CONCLUSION

The estimation of lamivudine and Tenofovir (TDF) was done by RP-HPLC. The mobile phase was optized Acetonitrile: water in the ratio of 55:45% v/v. A C18 column contains octa-decylsilane chemically linked to porous silica particles was used as stationary phase. UV detector was used at 262 nm. The solutions were chromatograph at a constant flow rate of 1 ml/min. The linearity range of lamivudine was found 6- 14µg/ml and tenofovir (TDF) were found to be 10-50µg/ml. Linear regression coefficient was not more than 0.999.

The results obtained on the validation parameters met ICH and USP requirements. It can be also inferred that the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

Table 11: Summary of developed method

Parameter	Lamivudine	Tenofovir
Linearity range (µg/ml)	6-14	10- 50
Regression coefficient (R^2)	0.999	0.999
% Recovery	99.47	99.60
Repeatability (n=6)	% RSD NMT 2	% RSD NMT 2
Precision		
Intraday precision	% RSD NMT 2	% RSD NMT 2
Interday precision		

FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Ms. Shweta Sharma performed experiment in the laboratory and collected data. Dr. Amar Deep Ankalgi analyzed and helps to perform the studies in laboratory and recorded observation and make necessary correction in the records. He also helps to design the experimental data and read the manuscript and make all the necessary corrections in manuscript. Miss. Pooja Kaushal analyzed the data and help to reading and drafting manuscript and help in research work. Dr. M.S. Ashawat studies all the records and helps to make necessary correction and approved the manuscript.

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