International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 1, 2016

Original Article

LIQUID CHROMATOGRAPHIC QUANTIFICATION OF TERNARY MIXTURE OF ANTI-VIRAL DRUGS AND APPLICATION TO ASSESSMENT OF THEIR TABLET DOSAGE FORM

ANUMOLU P. D., ANUSHA K., MRUDULA KIRAN A., MONIKA P., SOWNDARYA NSKR, SUNITHA G.*

Gokaraju Rangaraju College of Pharmacy, Department of Pharmaceutical Analysis and Quality Assurance, Osmania University, Hyderabad 500090, Telangana state, India
Email: g.sunitha88@gmail.com

Received: 17 Apr 2015 Revised and Accepted: 25 Nov 2015

ABSTRACT

Objective: To establish a validated liquid chromatographic method for the quantification of Tenfovir disoproxil fumarate (TENO), Lamivudine (LAM) and Nevirapine (NEV) in ternary combination and in its tablet dosage form.

Methods: The three drugs were well resolved using ODS C_{18} column (250 x 4.6 mm, 5µm) with a mobile phase consisting of phosphate buffer, pH 3.0-acetonitrile (60:40, v/v) with a flow rate of 1.0 ml/min at UV detection wavelength 252 nm. The developed method was validated as per ICH guidelines.

Results: The response was a linear function of analyte concentration over the concentration range of 45-105 µg/ml for tenofovir disoproxil fumarate, lamivudine and between 30-70 µg/ml for nevirapine with a correlation coefficient>0.9997. The % RSD values of precision and accuracy studies were found to be less than 2.

Conclusion: The proposed method was validated as per standard guidelines. The results obtained from assay values were in good agreement with the labeled amount of the marketed tablet dosage form RICOVIR-LN. The method holds promise for routine quality control of this ternary combination in bulk and pharmaceutical formulations.

Keywords: Column liquid chromatography, Tenofovir Disoproxil Fumarate, Lamivudine, Nevirapine.

INTRODUCTION

Tenofovir chemically designated as {[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl) oxy] methyl} phosphonic acid, is an anti-retroviral agent currently used in the treatment of HIV. Its mechanism of action involves the inhibition of HIV reverse transcriptase activity by competing with natural substrate deoxyadenosine5'-triphosphate by DNA chain termination. Nevirapine is chemically designated as 2-cyclopropyl-7-methyl- 2,4,9,15-tetra aza tricyclo [9.4.0.0^{3,8}] pentadeca-1(15)3,5,7,11,13-hexane-10-one, is an antiretroviral agent used in HIV treatment. Its mechanism of action involves binding of reverse transcriptase directly and blocks RNA-dependent and DNA–dependent polymerase activities by causing disruption of enzyme catalytic site. Lamivudine is also an anti-retroviral agent which is chemically designated as 4-amino-1-[(2R, 5S)-2-(hydromethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, used in the treatment of HIV. Chemical structures of these drugs were shown in fig.-1. The combination of three drugs used for the treatment of HIV as a ternary combination [1-2].

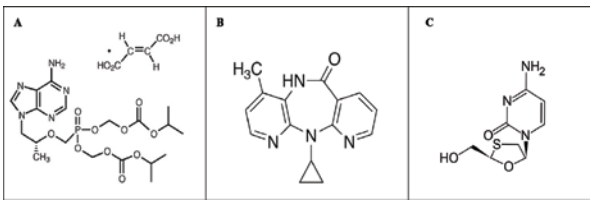

Fig. 1: Chemical structures of tenofovir disoproxil fumarate (A), Nevirapine (B) and Lamivudine (C)

An all-embracing literature search revealed that only one analytical method using high-performance liquid chromatography has been previously reported for the determination of this combination by gradient elution [3]. Few analytical methods based on HPLC, HPTLC, UV spectroscopy are available for the determination of these drugs individually and in combination with other drugs in different dosage forms, but there is no isocratic HPLC method for simultaneous quantification of three drugs in combined dosage form [4-14]. Keeping this point into consideration, present investigation has been undertaken with the objective to develop and validate simple isocratic RP-HPLC method for evaluation of this tertiary combination of drugs in bulk and tablet dosage form.

MATERIALS AND METHODS

Instrument and chromatographic conditions

The ternary combination of three drugs were well separated by using a Shimadzu model LC-20AD HPLC system connected to LC solutions software, equipped with an SPD-20A prominence UV detector and ODS, C_{18} column (250x4.6 mm, 5 µm) maintained at 25ºC. Isocratic elution was performed using acetonitrile and phosphate buffer, pH 3.0 (40:60, v/v) with flow rate 1.0 ml/min at UV detection wavelength 252 nm. 20 µl of sample was injected into the HPLC system.

Chemicals and reagents

Tenofovir disoproxil fumarate, nevirapine and lamivudine standards and tablet dosage forms were obtained from Mylan laboratories (Hyderabad, India). Milli-Q-water was used throughout the experiment. Acetonitrile and methanol (HPLC grade), sodium hydroxide, potassium dihydrogen phosphate, orthophosphoric acid were purchased from Merck (Mumbai, India).

Preparation of standard stock solution

The standard stock solution was prepared by dissolving 75 mg of tenofovir diisopropyl fumarate, 50 mg of nevirapine and 75 mg of lamivudine in 100 ml of mobile phase to get concentrations of 750 µg/ml, 500 µg/ml and 750 µg/ml respectively. This stock solution was further diluted with mobile phase to get the required concentration of drugs. Solutions were filtered through 0.22 µm membrane filter prior to injection into HPLC system.

Assay of commercial tablets

Twenty tablets of RICOVIR-LN were purchased, weighed and crushed into fine powder. An accurately weighed portion of tablet powder equivalent to 75 mg, 50 mg and 75 mg of tenofovir

disoproxil fumarate, nevirapine and lamivudine respectively was transferred into a 100 ml volumetric flask and made up to volume with mobile phase. The mixture was sonicated for 30 min and filtered to produce a final solution containing 75 µg/ml of tenofovir disoproxil fumarate, 50 µg/ml of nevirapine and 75 µg/ml of lamivudine respectively. The sample solution (20 µl) was injected into the HPLC system under optimized chromatographic conditions and peak area was measured from respective chromatograph to calculate the concentration of assay sample.

Method validation

Validation was done with respect to various parameters, as mentioned under ICH guidelines (ICH, Q2 (R1), 2005) [15-18].

Linearity

The linearity of the method was confirmed using a standard solution of the combination at different concentrations of analytes within the range of 45-105 µg/ml for tenofovir and lamivudine and between 30-70 µg/ml for nevirapine. Each solution was analyzed six times, and calibration plot was drawn by plotting the peak area against concentration.

Precision

Intra-day and inter-day precision were evaluated by determining the corresponding responses of standard solutions in six times on the same day and on different days (intermediate precision). The results were reported in terms of % RSD.

Accuracy

For the determination of % recovery, analysed samples were spiked with 80%, 100% & 120% level of standard drug and each solution was injected into HPLC system in six times and recovery was calculated by measuring peak areas.

Robustness

Robustness was studied to evaluate the effect of small but deliberate variations in the chromatographic conditions at different levels include wavelength (±2 nm), the percentage of acetonitrile (±10 %) and flow rate (±2 ml/min). One factor at a time was changed to estimate the effect.

LOD and LOQ

The limits of detection(LOD) and quantification (LOQ) were calculated by the method based on standard deviation(δ) of the response in triplicate and the slope (S) of the calibration plot, by use of formulae LOD=3.3δ/S and LOQ=10δ/S.

RESULTS AND DISCUSSION

The chromatographic conditions were optimized with a view to develop a reverse phase LC assay method. No internal standard was used, and no extraction or separation step was not involved. The optimization of the method parameters was done by fixing one variable and changing the other variables among mobile phase composition, flow rate and pH of the mobile phase. Tenofovir disoproxil fumarate, lamivudine, and nevirapine are partially soluble in water and freely soluble in organic solvents, such as methanol and acetonitrile. Among the different combinations for the solvent system, acetonitrile and phosphate buffer, pH 3.0, was preferred as the mixture, resulted in greater response to this drug combination after several preliminary investigatory runs. The pH of mobile phase was found to be critical because the peak obtained above pH 3.0 of buffer was irregular and asymmetric. Changes in the concentration of organic modifier often lead to significant changes in separation selectivity. Increasing in the buffer content in mobile phase resulted in a decrease in the retention time of the drugs. Therefore, a high buffer concentration was used at a flow rate of 1.0 ml/min. The peak shape and symmetry were found to be good when mobile phase composition of 60:40 (v/v) of phosphate buffer and acetonitrile was used at a flow rate of 1.0 ml/min at ambient temperature. Under these conditions, the analyte peak was well defined with very good symmetry, free from tailing and retention time of lamivudine, nevirapine, and tenofovir disoproxil fumarate was found to be at 2.350, 4.533 and 6.157 min respectively. The optimized chromatogram of proposed method was shown in fig. 2 and system suitability parameters reported in table 1. The method presents advantages over previously published methods by the usage of readily available mobile phase phosphate buffer and acetonitrile with UV-detector and short retention time enabled the analysis of a large number of samples with a small quantity of mobile phase, leading to its cost effectiveness. The results obtained from assay values were in good agreement with the labeled amount of the marketed tablet dosage form RICOVIR-LN (table 2).

Method validation

The correlation coefficient (R^2>0.9997) of the calibration plots indicated good linearity in the range of 45-105 µg/ml for lamivudine and tenofovir disoproxil fumarate and 30-70 µg/ml for nevirapine, and optimized conditions of proposed method showed in table 3. No significant difference between intra-day and inter-day precision values revealed that the method was reproducible (table-4). The % recovery of the method ranged from 98-102 after spiking previously analysed samples with 80%, 100% and 120% of additional drug and % RSD values less than 2 indicates the method is accurate (table-5).

The robustness of the method was investigated under a variety of conditions with respect to changes in flow rate, wavelength, and composition of the mobile phase. The results revealed that the method is robust for normally expected variations in chromatographic conditions (table 6). The LOD for lamivudine, nevirapine, and tenofovir disoproxil fumarate were 1.95, 1.26 and 2.91 µg/ml respectively. The LOQ values of lamivudine, nevirapine, and tenofovir disoproxil fumarate were found to be 5.92, 3.81 and 8.83 µg/ml. The results strongly advocate that developed method is sensitive.

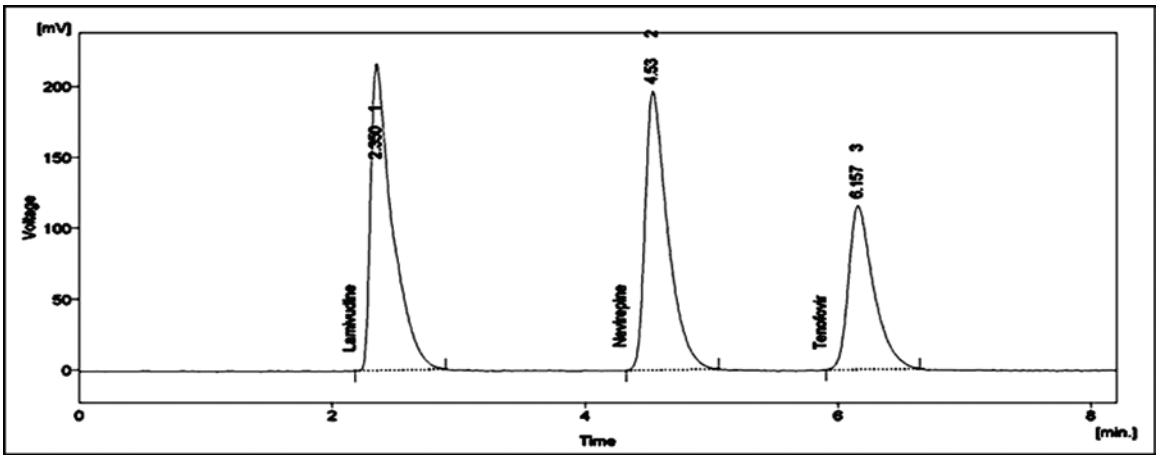

Fig. 2: Optimized chromatogram of the proposed method

Table 1: System suitability parameters for the proposed method

Parameter	LAM	NEV	TEN
Retention time (min)	2.350	4.533	6.157
Theoretical plates	31173	65349	98331
Tailing factor	1.640	0.412	1.023

Table 2: Assay for the proposed method

S formulation		Label claim (mg)	Amount found (mg)	% assay*
RICOVIR-LN	LAM	300	299.65	99.88±0.024
	NEV	200	199.13	99.57±0.124
	TEN	300	297.19	99.07±0.254

*Mean of three replicates

Table 3: Analytical parameters for the determination of the ternary drug combinations using proposed RP-HPLC method

Parameters	Analyte		
	LAM	NEV	TEN
Wavelength (nm)	252	252	252
Concentration Range (µg/ml)	45-105	30-70	45-105
Intercept*	-65.67	+16.75	-47.35
Slope*	37.86	51.04	25.30
Standard deviation*	23.7	19.235	23.717
% RSD*	0.975	0.7936	1.5086
Correlation coefficient	0.9998	0.999	0.997
LOD (µg/ml)	1.95	1.26	2.91
LOQ (µg/ml)	5.92	3.81	8.83

*****Mean of six determinations

Table 4: Precision data

Parameters	Analyte (% RSD *)		
	LAM	NEV	TENO
Intra-day precision	0.29	0.39	1.12
Inter-day precision	0.243	0.391	1.001

*Mean of three determinations

Table 5: Recovery data

Formulation	Mixture	Levels (%)	Theoretical (mg)	Added (mg)	Measured* (mg)	Recovery (%)	%RSD*
		80		20	45.65	101.44	0.291
RICOVIR-LN	LAM	100	25	25	50.57	101.10	0.526
		120		30	56.01	101.83	0.336
		80		24	54.81	101.52	0.253
	NEV	100	30	30	61.02	101.70	0.633
		120		36	67.12	101.61	0.516
		80		20	44.19	98.22	0.712
	TENO	100	25	25	50.82	101.64	0.625
		120		30	56.35	100.60	0.512

*Mean of three replicates.

Table 6: Robustness for the proposed method

Parameter	Deliberate change	Retention time*			Area*		
		TEN	NEV	LAM	TEN	NEV	LAM
Flow rate	0.8	8.357	6.0166	3.706	1966.98	3043.70	3097.66
	1.2	5.5866	4.045	2.497	1311.07	2035.07	2037.60
Wavelength	250	8.357	6.016	3.706	1966.9	3043.7	3097.6
	254	5.586	4.045	2.497	1311.0	2035.0	2037.6
Mobile phase	-10 %	5.586	4.045	2.497	1311.07	2037.07	2037.6
	+10 %	8.357	6.016	3.706	1966.98	3043.70	3097.66

*Average of three replicates

CONCLUSION

Literature survey revealed that isocratic RP-HPLC method was not available for simultaneous quantification of lamivudine, nevirapine and tenofovir diisopropyl fumarate in ternary mixture and tablet dosage form. In present proposed method, the complete separation of three analytes was accomplished in less than 7 min with same mobile phase composition throughout the analysis, revealed that the proposed method is economic and simple than previous gradient method. The proposed method was validated as per standard guidelines and results obtained from validation proved that the method was specific, precise, accurate and robust. The developed method was also utilized for assay of commercial tablets, and obtained values are good agreement with their labeled claim. These advantageous encourage that; the developed method can be routine quality control of this ternary combination in bulk and pharmaceutical formulations.

ACKNOWLEDGEMENT

The authors are thankful to the management and Prof. C. V. S. Subramanyam, Principal, Gokaraju Rangaraju College of Pharmacy for supporting this work.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Maryadele, O'Neil J. An encyclopedia of chemicals, drugs, and biological. 14th ed. Merck research laboratories, Division of Merck and co., Inc, White house station, NJ, USA; 2006. p. 927, 1123, 1572.
2. Goodman AG, Gilman LS. The pharmacological basis of therapeutics. 9th ed. Hardman, Limbird JG, LE, Eds. McGraw-Hill Companies, Hightstown, NJ; 1996.
3. Lanka A, Prasad S. New stability indicating a method for simultaneous quantification of related impurities of Lamivudine, Tenofovir, and Nevirapine in extended release tablet dosage forms. Int Res J Pharm 2012;3:103-8.
4. Ashok G, Jahnavi B. Method development and validation for the simultaneous estimation of Lamivudine and Tenofovir disoproxil fumarate in pharmaceutical dosage forms by RP-HPLC. Int J Adv Res Pharm Bio Sci 2013;2:22-32.
5. Sonawane PH, Panzade PS, Kale MA. Simultaneous estimation of lamivudine and tenofovir disoproxil fumarate in bulk and combined pharmaceutical dosage form by HPLC method. Asian J Biomed Pharm Sci 2013;3:27-30.
6. Chandra P, Rathore AS, Mahadik KR. Application of the high-performance thin-layer chromatographic method for the simultaneous determination of Lamivudine and Tenofovir disoproxil fumarate in pharmaceutical dosage form. J Chil Chem Soc 2011;56:702-5.
7. Padma A, Raghuveer P, Swaroopa K, Shravani O. A validated RP-HPLC method for estimation of Tenofovir Disoproxil Fumarate in tablet dosage form and its stress degradation studies. Asian Pharma Press 2011;4:4426.
8. Shweta H, Sunil R. Development and validation of a stability-indicating LC method for the determination of tenofovir disoproxil fumarate in a pharmaceutical formulation. Songklanakarin J Sci Technol 2012;34:615-22.
9. Tripti S, Neelam M, Swapank M, Sankar G. A validated RP-HPLC method for estimation of Tenofovir disoproxil fumarate in bulk and pharmaceutical formulation. Asian J Pharm Clin Res 2012;5:108-10.
10. Jayaseelan S, Suresh S, Sathishkumar G, Sekar V, Perumal P. Bioanalytical method development and validation of Lamivudine by RP-HPLC method. Int J ChemTech Res 2010;2:163-7.
11. Akhilesh V, Singha A. Development and validation of analytical method for the estimation of Lamivudine in rabbit plasma. J Pharm Anal 2011;1:251-7.
12. Prasada Rao CH, Channabasavaraj KP, Lakshmi Aswini G. Development and validation of RP-HPLC method for the estimation of Nevirapine in bulk drug and tablets. J Pharm Sci Res 2009;1:78-82.
13. Sindhu Reddy M, Ramya Tulasi G, Neeharika G, Swetha M, Agilandeswari D, Dabhi Harshkumar D. Method development and validation of Neverapine by RP-HPLC. Pharm Innovation 2012;1:8-13.
14. Mandloi DK, Rai VK, Dey S, Tyagi PK, Ashada RK, Mohanraj P, e. Method development and validation of RP-HPLC method in the detection of Nevirapine in bulk drug and tablet formulation and application to *in-vitro* dissolution study. J Glob Pharm Tech 2009;1:54-61.
15. International Conference on Harmonization. Validation of Analytical Procedures: Text and Methodolgy ICH Q2B (R1); 2005.
16. Alapati VR, Divya TG, Sunitha G, Rajesh CH, Vamshikrishna L, Panikumar AD. Validated stability indicating a liquid chromatographic method for quantification of Ciprofloxacin HCl, its related substance, and tinidazole in tablet dosage form. Int J Pharm Pharm Sci 2014;6:611-5.
17. Venkata RY, Sunitha G, Ashish KP, Haripriya A, Sirisha N, Panikumar AD. Validated stability indicating the analytical method and in-vitro dissolution studies of Efavirenz formulation by RP-HPLC. Int J Pharm Pharm Sci 2012;4:572-6.
18. Senthilkumar KR, Meyyanathan SN, Gowramma B. A new validated chiral RP-HPLC method for the determination of stability of Mebeverine enantiomers in the presence of its degradation products. Int J Pharm Pharm Sci 2015;7:112-6.