



DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR AND EFAVIRENZ BY UPLC

*Sk. Madeesh, Y. Ismail and V. Gunasekaran

Rao's College of Pharmacy, Nellore, Andhra Pradesh, India

*Corresponding author e-mail: madeeshpharma@gmail.com

ABSTRACT

A simple, accurate, rapid and precise isocratic reversed-phase high-performance liquid chromatographic method has been developed and validated for simultaneous determination of lamivudine, tenofovir and efavirenz in tablets. The chromatographic separation was carried out on a BEH symmetry C₁₈ (50×4.6mm, 1.7 μm) column with a mixture of methanol: phosphate buffer pH 3.0 adjusted with *o*-phosphoric acid (65:35, v/v) as mobile phase; at a flow rate of 0.3 mL/min. The retention times for LAM, TEN and EFA were observed to be 0.432, 0.657, 2.281 min, respectively. Calibration plots were linear ($r^2 > 0.999$) over the concentration range of 10-50 μg/mL for LAM and TEN; and 20-100 μg/mL for EFA. The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The proposed method was successfully used for quantitative analysis of tablets. No interference from any component of pharmaceutical dosage form was observed. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for routine determination of lamivudine, tenofovir and efavirenz in tablets.

Keywords: Lamivudine, Tenofovir, efavirenz and UPLC

INTRODUCTION

Multi-drug antiretroviral therapy has resulted in a significant improvement of the health condition of acquired immunodeficiency syndrome (AIDS). The multi-drug combinations of nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors are effective in the therapy of human immunodeficiency virus (HIV) infection. It is used as a part of highly active anti retroviral therapy (HAART), for the treatment of HIV-1^[1,2].

Efavirenz (EFV), (4S)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2H-3,1-benzoxazin-2-one, is an antiretroviral drug which is a non-nucleoside reverse transcriptase inhibitor (NNRTI)^[3,4]. EFV has been determined by UV spectroscopic^[5] and RP-HPLC^[6] methods in single and in combined dosage form. Tenofovir disoproxil fumarate (TDF), 9-((R)-2-((bis((isopropoxycarbonyl)oxy)methoxy)phosphinyl)methoxy)propyl)adenine fumarate (1:1), is a nucleotide analogue reverse transcriptase inhibitor

(nRTIs)^[3,4]. TDF has been determined in spiked human plasma by HPLC^[7,8]. The estimation of TDF by RP-HPLC has been reported^[6,9]. Lamivudine (LMI), (2R,cis)-4-amino-1-(2-(hydroxymethyl)-1,3-oxathiolan-5-yl)-(1H) pyrimidin-2-one, is nucleoside-reverse transcriptase inhibitor (NRTI)^[3,4]. It is an analogue of cytidine. The estimation of lamivudine using UV^[5,10-12] spectroscopy and HPLC has been reported^[11,13]. From literature, no UPLC method has been reported for the simultaneous estimation of lamivudine, tenofovir and efavirenz. Hence, a rapid, precise and accurate method for the quantification of efavirenz, tenofovir and lamivudine in pharmaceutical formulations is developed and validated.

MATERIALS AND METHODS

Chemicals and reagents: Pharmaceutical grade of LAM, TEN and EFA were kindly supplied as gift samples by Torrent Pharmaceuticals, Gujarat, India, certified to contain > 99% (w/w) on dried basis.

Commercially available TRIODAY (Cipla Ltd., Mumbai, India) tablets claimed to contain 300 mg LAM; 300 mg TEN and 600 mg EFA have been utilized in the present work. All chemicals and reagents used were of HPLC grade and were purchased from Merck Chemicals, India.

Chromatographic system and conditions: A Waters UPLC system consisting of a Water H05UPB062M binary gradient pump, an inbuilt auto sampler, a column oven and Water 2996 PDA detector was employed throughout the analysis. The data was acquired using Empower 2 software. The column used was BEH symmetry C₁₈ (50×4.6mm, 1.7 μm). A Bandline sonerex sonicator was used for enhancing dissolution of the compounds. A Digisum DI 707 digital pH meter was used for pH adjustment.

The mobile phase consisted of methanol and potassium phosphate buffer adjusted to pH 3.0 with *o*-phosphoric acid in the ratio 65:35 (v/v). Before analysis the mobile phase was filtered through a 0.45 μ filter and degassed by ultrasonification. The flow rate was 0.3 mL/min. Detection was monitored at 260 nm and injection volume was 6 μL. All the experiments were performed at ambient temperature.

Standard solutions and calibration graphs for chromatographic measurement: Stock standard solutions were prepared by dissolving separately 10 mg of LAM, TEN and EFA in 10 ml methanol (1000 μg/mL). The standard calibration solutions were prepared by appropriate dilution of the stock solution with mobile phase to reach a concentration range of 10-50 μg/mL for LAM and TEN and 20-100 μg/mL for EFA. Triplicate 6 μL injections were made for each concentration and chromatographed under the optimized conditions described above. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Sample preparation: Twenty tablet contents were accurately weighed, their mean weight was determined and they were mixed and finely powdered. A portion equivalent to about one tablet was accurately weighed and transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol.

The resulting solution was centrifuged at 3000 rpm for 5 min. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45 μ filter (Millipore, Milford, MA). The original stock solution was further diluted to get sample solution of drug concentration of 30 μg/mL for LAM

and TEN and 60 μg/mL EFA. A 6 μL volume of sample solution was injected into UPLC system, six times. The peak areas for the drugs were measured at 260 nm and concentration in the sample was determined by comparing the area of sample with that of the standard.

Method validation ^[14]: The developed method was validated according to the ICH guidelines. The system suitability was evaluated by six replicate analyses of LAM, TEN and EFA mixture at a concentration of 30 μg/mL for LAM and TEN and 60 μg/mL EFA. The acceptance criteria were a %RSD of peak areas and retention times less than 2, theoretical plate numbers (N) at least 2500 for each peak and tailing factors (T) less than 1% for LAM, TEN and EFA.

Standard calibration curves were prepared in the mobile phase with six concentrations ranging from 10-50 μg/mL for LAM and TEN; and 20-100 μg/mL for EFA in triplicate into the UPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Limit of detection (LOD) and limit of quantization (LOQ) were estimated from the signal-to-noise ratio. LOD and LOQ were calculated using $3.3\sigma/s$ and $10\sigma/s$ formulae, respectively, where, σ is the standard deviation of the peak areas and s is the slope of the corresponding calibration curve.

To study the reliability and suitability of the developed method, recovery experiments were carried out at three levels 50, 100 and 150%. Known concentrations of commercial tablets were spiked with known amounts of LAM, TEN and EFA. The accuracy was expressed as the percentage of analyte recovered by the proposed method.

Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of LAM, TEN and EFA were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). Every sample was injected in triplicate. The repeatability of sample application and measurement of peak area for active compounds were expressed in terms of percent RSD.

To evaluate robustness of HPLC method a few parameters were deliberately varied. The parameters included variation of flow rate, percentage of buffer in the mobile phase, and pH of mobile phase. All

chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks. Marketed formulations were analyzed to determine the specificity of the optimized method in the presence of common tablet excipients.

RESULTS AND DISCUSSION

During the optimization of UPLC method, two organic solvents (acetonitrile and methanol), two buffers (acetate and phosphate) at two different pH values (3 and 4) were tested. Initially methanol: water, acetonitrile: water, acetonitrile: acetate buffer, methanol: acetate buffer were tried in different ratios at pH 3 and 4. ASP and ATO eluted with the tried mobile phases, but CLO was retained. Then, with acetonitrile: phosphate buffer all the three drugs eluted, but the analysis time was more than 15 min. In order to decrease the analysis time, column length was reduced from 150 to 50 mm.

The mobile phase conditions were optimized so the peak from the first-eluting compound did not interfere with those from the solvent, excipients. Other criteria, *viz.* time required for analysis, appropriate *k* range ($1 < k < 10$) for eluted peaks, assay sensitivity, solvent noise were also considered.

Finally a mobile phase consisting of a mixture of methanol: phosphate buffer pH 3.0 adjusted with *o*-phosphoric acid in ratio 65:35 (v/v), was selected as mobile phase to achieve maximum separation and sensitivity. Flow rates between 0.2 to 0.8 mL/min were studied. A flow rate of 0.3 mL/min gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed phase C₁₈ column, the retention times for LAM, TEN and EFA were observed to be 0.432, 0.657, 2.281 min, respectively. Total time of analysis was less than 6 min. The chromatogram at 260 nm showed a complete resolution of all peaks (Figure 1).

Validity of the analytical procedure as well as the resolution between different peaks of interest is ensured by the system suitability test. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatogram, all three analytes are eluted by forming symmetrical single peaks well separated from the solvent front. Excellent linearity was obtained for all the three drugs in the range of 10-50 µg/mL for LAM and TEN; and 20-100 µg/mL for EFA. The correlation coefficients (r^2) were found to be greater than 0.999 ($n=6$) in all

instances. The results of calibration studies are summarized in (Table 1).

The proposed method afforded high recoveries for LAM, TEN and EFA tablets. Results obtained from recovery studies presented in (Table 2), indicate that this assay procedure can be used for routine quality control analysis of this ternary mixture in tablets.

Precision of the analytical method was found to be reliable based on % RSD ($< 2\%$) corresponding to the peak areas and retention times. The % RSD values were less than 2, for intra-day and inter-day precision. Hence, the method was found to be precise for all the three drugs.

The chromatograms were checked for the appearance of any extra peaks. It was observed that single peak for LAM ($R_t \pm SD$, 0.437 \pm 0.01), TEN ($R_t \pm SD$, 0.661 \pm 0.21) and EFA ($R_t \pm SD$, 2.286 \pm 0.15) were obtained under optimized conditions, showing no interference from common capsule excipients and impurities. Also the peak areas were compared with the standard and % purity calculated was found to be within the limits.

LOD and LOQ were found to be 0.004 µg/mL and 0.015 µg/mL for LAM, 0.006 µg/mL and 0.03 µg/mL for TEN and 0.03 µg/mL and 0.1 µg/mL for EFA. In all deliberately varied conditions, the SD of retention times of LAM, TEN and EFA were found to be well within the acceptable limit. The tailing factor for all the three peaks was found to be < 1.5 .

The validated method was used in the analysis of marketed conventional TRIODAY tablets claimed to contain 300 mg LAM; 300 mg TEN and 600 mg EFA. Representative chromatogram is shown in (Figure 2). The results for the drugs assay show a good agreement with the label claims (Table 3).

CONCLUSION

The developed UPLC method is simple, specific, accurate and precise for the simultaneous determination of LAM, TEN and EFA from pharmaceutical formulations. The developed method provides good resolution between LAM, TEN and EFA. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness in accordance with ICH guidelines. Thus, the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination.

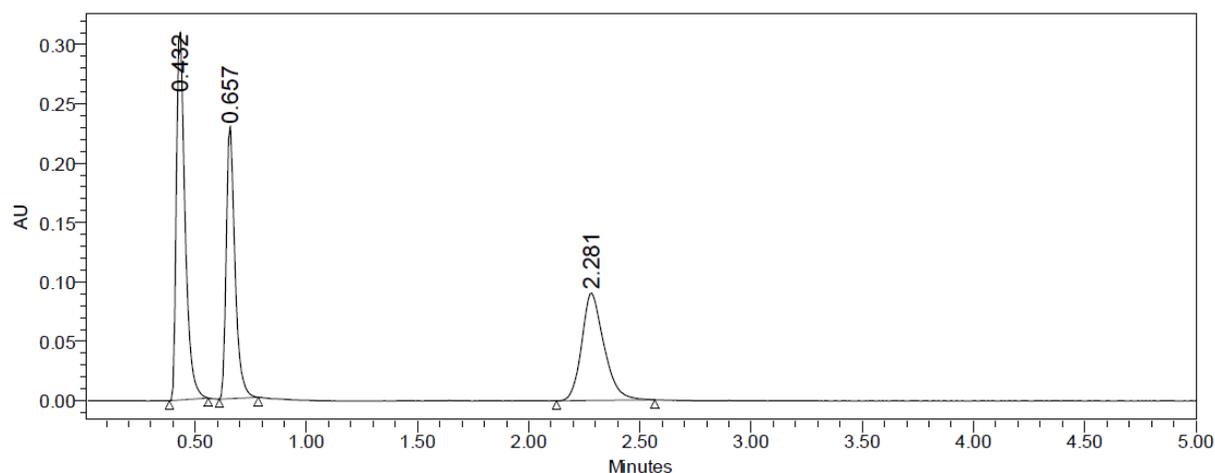


Figure 1: A typical standard chromatogram of lamivudine (0.432), tenofovir (0.657) and Efavirenz (2.281)

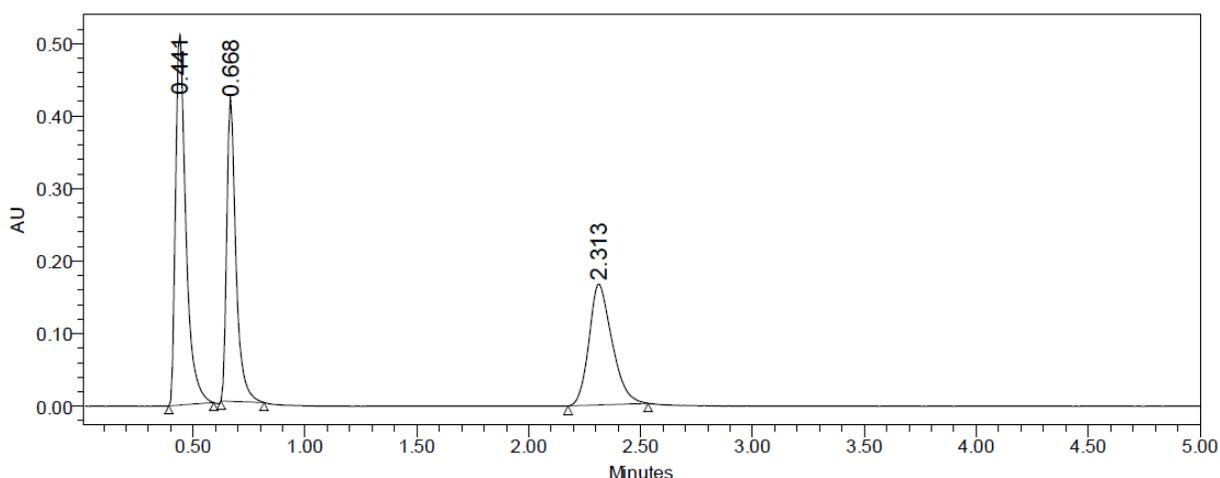


Figure 2: A typical chromatogram of tablet contains lamivudine (0.441), tenofovir (0.668) and Efavirenz (2.313)

Table 1: Linearity parameters for simultaneous estimation of lamivudine, tenofovir and Efavirenz

Parameters	Lamivudine	Tenofovir	Efavirenz
Linearity range ($\mu\text{g/mL}$)	10-50	10-50	20-100
r^2 value	0.9991	0.9995	0.9991
LOD ($\mu\text{g/mL}$)	0.004	0.006	0.03
LOQ ($\mu\text{g/mL}$)	0.015	0.03	0.10

Table 2: Recovery studies

Accuracy level	Mean recovery (% Mean \pm SD; %RSD)		
	LAM	TEN	EFA
At 50%	99.83 \pm 0.862; 0.863	100.46 \pm 1.091; 1.086	99.79 \pm 0.671; 0.672
At 100%	101.08 \pm 0.805; 0.796	99.33 \pm 1.135; 1.143	100.61 \pm 0.659; 0.655
At 150%	98.39 \pm 0.228; 0.232	100.35 \pm 1.137; 1.133	101.02 \pm 1.075; 1.064

Table 3: Assay results of lamivudine, tenofovir and efavirenz in marketed formulation

Drug	% Amount found±SD	%RSD	SEM
Lamivudine 300 mg	99.76±0.887	0.889	0.3619
Tenofovir 300 mg	98.88±0.842	0.851	0.3437
Efavirenz 600 mg	99.13±0.883	0.891	0.3606

REFERENCES

1. World Health Organization says failure to deliver AIDS medicines is a global health emergency. WHO Press Release September 2003.
2. Rouzes A, et al. J Chromatogr B, 2004; 813: 209-16.
3. The Merck Index. In: Budawari S, editor. 13th ed. Whitehouse Station, NJ: Merck and Co Inc; 2001.
4. Martindale: The complete drug reference. In: Sweetman SC, editors. 33rd ed. London: The Pharmaceutical Press; 2002.
5. Nagori BP, Kumar P. Indian Drugs, 2008; 45: 558-62.
6. Mangaonkar K, Desai A. Indian Drugs, 2008; 45: 188-92.
7. Sentenac S, Fernandez C, Thuillier A, Lechat P, Aymard G. J Chromatogr B Analyt Technol Biomed Life Sci, 2001; 793: 317-24.
8. Kandagal PB, Manjunatha DH, Seetharamappa J, Kalanur SS. Anal Lett, 2008; 41: 561-70.
9. Mangaonkar K, Desai A. Indian Drugs, 2008; 45: 119-22.
10. Uslu B, Özkan SA. Anal Chim Acta, 2002; 466: 175-85.
11. Kapoor N, Khandavilli S, Panchagnula R. J Pharm Biomed Anal, 2006; 41: 761-5.
12. Sankar G, Reddy MV, Rajendra Kumar JM, Murthy TK. Indian J Pharm Sci, 2002; 64: 504-6.
13. Palled MS, Rajesh PM, Chatter M, Bhat AR. Indian J Pharm Sci, 2005; 67:110-2.
14. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedure: Methodology (ICH – Q 2B) November 1996; 1-8.