



Development and Validation of Stability-Indicating Thin layer Chromatographic-Densitometric Determination of Abacavir, Lamivudine and Zidovudine in Bulk and Pharmaceutical Dosage Form

Charushila H. Bhirud*, Shivanand N.Hiremath

*Ph.D. Scholar, Pravara Institute of Medical Sciences Deemed University (PIMS), Loni, Maharashtra, India.

Principal, Pravara Rural College of Pharmacy for Women, Chincholi, Nasik, Maharashtra, India.

*Corresponding author's E-mail: bhangale100@rediffmail.com

Accepted on: 07-05-2013; Finalized on: 31-07-2013.

ABSTRACT

A simple, selective, precise and stability-indicating high-performance thin-layer chromatographic method for densitometric determination of Abacavir, Lamivudine and zidovudine in both as a bulk drug and in formulation was developed and validated as per the International Conference on Harmonization guidelines. The method employed TLC aluminium plates precoated with silica gel 60F 254 as the stationary phase. The solvent system consisted of Toluene: Ethyl acetate: methanol (8.0:1.0:1.0 v/v/v). Densitometric measurement of Abacavir, Lamivudine and zidovudine was performed in the absorbance mode at 279 nm. The system was found to give compact spot for Abacavir, Lamivudine and zidovudine (R_f value of 0.26, 0.46, 0.65). The calibration curve of the drug was linear in the range of 500-4000 ng/spot for Abacavir with $r^2 = 0.9995 \pm 0.0001$, 200-1200 ng/spot for Lamivudine with $r^2 = 0.9990$ and 300-1800 ng/spot for Zidovudine with $r^2 = 0.9994$. The mean value \pm SD of slope and intercept were 3.436 ± 0.039 and 763.16 ± 62.401 for Abacavir, 2.186 ± 0.012 and 326.06 ± 45.421 for lamivudine and 1.236 ± 0.12 and 123.12 ± 23.213 for zidovudine with respect to peak area respectively. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of said drug. As the proposed method can effectively separate the drug from its degradation products, it can be employed as stability-indicating method.

Keywords: Abacavir, Degradation, HPTLC, Lamivudine, Stability-indicating, Validation, Zidovudine.

INTRODUCTION

Abacavir, lamivudine and zidovudine are synthetic nucleoside analogs showing a potent and synergistic effect on inhibition of the human immunodeficiency virus (HIV-1), the causative agent of acquired immuno-deficiency syndrome (AIDS).¹ Abacavir (ABV) is [1R]-4-(2-amino-6-cyclopyridine) purine-9-yl)-1-cyclopent-2-enyl] methanol. It is a nucleoside reverse transcriptase inhibitor (NRTI) with an activity against human immune deficiency virus Type (1) (HIV1).² Lamivudine (LMV) is 4-amino-1-[2R, 5S]-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl] pyrimidine-2-one. Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTI) with an activity against human immunodeficiency virus type (1) (HIV) 1 and hepatitis B.³ Zidovudine (ZDV) is (3'-azido-3'-deoxythymidine).⁴ The chemical structures of drugs are shown in Figure 1.

Literature survey revealed few Spectrophotometric⁵⁻⁸ and chromatographic⁹⁻¹⁶ methods for estimation of ABV, LMV and ZDV in pharmaceutical formulation and biological fluids has been reported.

However, to our knowledge, no information related to the stability-indicating high-performance thin-layer chromatography (HPTLC) determination of ABV, LMV and ZDV in pharmaceutical dosage forms has ever been mentioned in literature. The parent drug stability test guidelines (Q1A) issued by International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability indicating.¹⁷⁻¹⁹

The present paper describes a reliable, rapid and accurate stability-indicating HPTLC method for determination of ABV, LMV and ZDV using HPTLC densitometry.

Hence, an attempt has been made to develop an accurate, specific and reproducible method for the determination of ABV, LMV and ZDV in presence of degradation product for the content analysis during stability studies from pharmaceutical dosage form

MATERIALS AND METHODS

Reagents

ABV, LMV and ZDV were kindly supplied as a gift sample by Hetero Lab., India. All the reagents used were of analytical reagent grade (S.D. Fine Chemicals, Mumbai, India) and used without further purification.

Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of width 6 mm with 100 μ L sample syringe on precoated silica gel aluminium plate 60 F254 (20 cm \times 10 cm) with 250 μ m thickness; (E MERCK, Darmstadt, Germany) using a Camag Linomat V (Switzerland). The plates were prewashed with methanol and activated at 110°C for 5 min, prior to chromatography. A constant application rate of 150 nl/sec was employed and space between two bands was 15.4 mm. The slit dimension was kept at 6 mm \times 0.45 mm. The mobile phase consists of Toluene: Ethyl acetate: methanol (8.0:1.0:1.0 v/v/v). Linear ascending development was carried out in 20 cm \times 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was

20 min, at temperature ($25^{\circ}\text{C} \pm 2$); the relative humidity ($60\% \pm 5\%$); the length of chromatogram run was 8 cm and TLC plates were air dried. Densitometric scanning was performed on Camag TLC Scanner 3 equipped with

winCATS software version 1.3.0 at 276 nm. The source of radiation utilized was deuterium lamp. Evaluation was performed using peak area with linear regression.

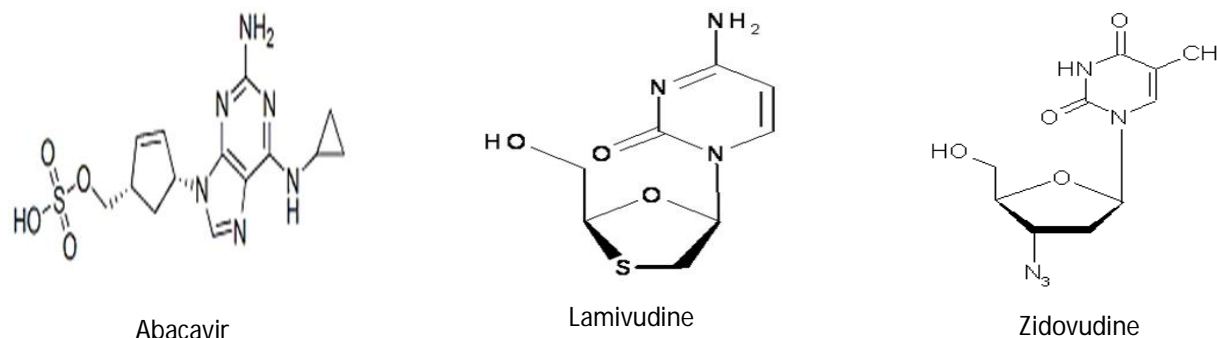


Figure 1: Structure of Abacavir, Lamivudine and Zidovudine

Standard solutions and calibration graphs

Combined standard stock solution containing 1000 $\mu\text{g}/\text{ml}$ of ABV, 500 $\mu\text{g}/\text{ml}$ of LMV and 1000 $\mu\text{g}/\text{ml}$ of ZDV was prepared in methanol. Calibration was done by Hamilton syringe with the help of automatic sample applicator Linomat V on TLC plate that gave concentration 500 -4000 ng/spot of ABV, 200 - 1200 ng/spot of LMV and 300-1800 ng/spot for ZDV respectively. Each concentration was spotted six times on the TLC plates. The plates were developed using previously described mobile phase. The calibration graph was plotted as peak areas versus corresponding concentrations.

Method validation

Precision

Repeatability of sample application was assessed by spotting 1000 ng/spot for ABV, 500 ng/spot for LMV and 1000 ng/spot for ZDV of drug solution six times on a TLC, followed by development of plate. The intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing drug solutions within the calibration range on three different days over a period of a week.

Limit of detection and limit of quantification

In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. Stock solution of ABV, LMV and ZDV were prepared and different volume of stock solution in the range 500 to 1000 ng for ABV, 200 to 300 ng for LMV and 300-600 ng for ZDV were spotted in triplicate. The amount of both the drugs by spot versus average response (peak area) was graphed and the equation for this was determined. The standard deviations (S.D.) of responses were calculated. The average of standard deviations was calculated (A.S.D.). Detection limit was calculated by $(3.3 \times \text{A.S.D.})/b$ and quantification limit was calculated by $(10 \times \text{A.S.D.})/b$, where "b" corresponds to the slope obtained in the linearity study of method.

Specificity

Specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs very efficiently. The spot for ABV, LMV and ZDV were confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of ABV, LMV and ZDV were assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

Accuracy

Recovery study was carried out by over spotting 80%, 100% and 120% of the standard drug solution of ABV, LMV and ZDV and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check the recovery of the drug at different levels in formulation.

Robustness

Robustness was studied in six replicate at the concentration level of 1000 ng/spot for ABV, 500 ng/spot for LMV and 1000 ng/spot for ZDV. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography and chromatography to spotting) were studied and the effects on the results were examined.

Ruggedness

The ruggedness of the proposed method was evaluated by two different analysts.

Analysis of ABV, LMV and ZDV in marketed formulation

To determine the content of ABV, LMV and ZDV simultaneously in conventional tablets (label claim 300 mg ABV, 150 mg LMV and 300 mg ZDV); twenty tablets were accurately weighed, average weight determined and ground to a fine powder. A quantity of powder equivalent to 200 mg ABV, 100 mg of LMV and 200 mg ZDV was transferred into 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to mark with same solvent. The resulting solution was filtered using

0.45 μm filter (Millifilter, MA). 0.4 μL of the above solution applied on TLC plate followed by development and scanning as described in section 2.2. The analysis was repeated for six times. ABV, LMV and ZDV gave sharp and well defined peaks at R_f 0.26, 0.46 and 0.65, respectively, when scanned at 279 nm. The results are shown in Table 1 indicate that there was no interferences from the excipients commonly present in the tablets.

Table 1: Assay of tablet formulation

Component	Label Claim (mg)	% Label Claim*	% RSD
ABV	300	100.89	0.59
LMV	150	100.84	0.56
ZDV	300	100.23	0.63

*mean of six estimations

Forced degradation of ABV, LMV and ZDV

Acid and base induced degradation

The 10 mg of ABV, LMV and ZDV were separately dissolved in 10 ml methanolic solution of 1 M HCl and 1 M NaOH. These solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The 1 ml of above solutions were taken, neutralized and diluted up to 10 ml with methanol. The resultant solution were applied on TLC plates in triplicates (10 μl each, i.e. 1000 ng/spot). The chromatograms were run as described in section 2.2.

Hydrogen peroxide – induced degradation

The 10 mg of ABV, LMV and ZDV were separately dissolved in 10 ml of methanolic solution of hydrogen peroxide (10%, v/v). The solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The 1 ml of above solution were taken and diluted up to 10 ml with methanol. The resultant solution were applied on TLC plate in triplicate (10 μl each, i.e. 1000 ng/spot). The chromatograms were run as described in section 2.2.

Dry heat degradation products

ABV 10 mg, LMV 10 mg and ZDV 10 mg were stored at 55^o C for 3 h in oven separately. They were transferred to 10 ml volumetric flask containing methanol and volume was made up to the mark. 10 μl (1000 ng / spot) was applied on TLC plate in triplicate and chromatogram was run as described in section 2.2.

Light heat degradation products

The 10 mg of ABV, LMV and ZDV were dissolved in 10 ml of methanol separately. The solutions were kept in the sun light for 8 h. The 1 ml of above solutions were taken and diluted up to 10 ml with methanol. The resultant solutions were applied on TLC plate in triplicate (10 μl each, i.e. 1000 ng / spot). The chromatograms were run as described in section 2.2.

RESULTS AND DISCUSSION

Optimization of HPTLC method

Initially, toluene: ethyl acetate: methanol in the ratio 4: 2: 2 (v/v/v) was tried for both drugs simultaneously. The spots were not developed properly and dragging was observed. Then, toluene: ethyl acetate: methanol in the ratio of 6: 4: 3 (v/v/v) was tried. The developed spots were diffused. Finally, mobile phase consisting of toluene: ethyl acetate: methanol: acetic acid (8:1:1 v/v/v) gave good resolution (Figure 2).

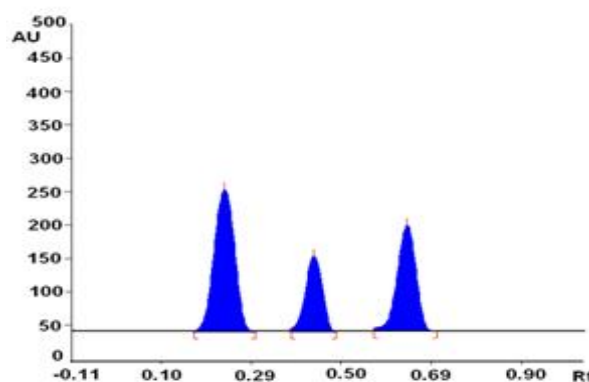


Figure 2: Typical HPTLC chromatogram of ABV, LMV and ZDV (R_f -0.26, 0.46 and 0.65 respectively)

Both the peaks were symmetrical in nature and no tailing was observed when plate was scanned at 279 nm. The chamber was saturated with the mobile phase for 20 min at room temperature and plates were activated at 110^o C for 5 min to obtain well-defined spots.

Linearity

Linearity responses for ABV, LMV and ZDV were assessed in the concentration ranges 500 - 4000ng /spot, 200-1200 ng /spot and 300-1800 ng /spot, respectively. The linear equations for the calibration plots were $Y = 2.6712 X + 1161.1$, $Y = 8.0837 + 25.859$ and $Y = 4.4562 + 234.23$, with correlation coefficient (r) being 0.9995, 0.9990 and 0.9994 for ABV, LMV and ZDV, respectively. Range was established with five replicate readings of each concentration.

Validation of the method

Precision

Precision of the method was determined in the terms of intra-day and inter-day variation (%RSD). Intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day.

Inter-day precision (%RSD) was assessed by analyzing drug solutions within the calibration range on three different days over a period of a week.

Limit of detection and limit of quantitation

Detection limit and quantification limit was calculated by the method as described in section 2.4.2 The LOQ and LOD for ABV were 42.40 ng and 13.99 ng. For LMV, LOQ

and LOD were found to be 22.32 ng and 7.37 ng, and for ZDV were found to be 15.23 and 7.23 ng respectively. This indicates that adequate sensitivity of the method.

Accuracy

To the pre-analyzed sample a known amount of standard solution of pure drug (ABV, LMV and ZDV) was over spotted at three different levels i.e. at 80 %, 100% and 120%. These solutions were subjected to re-analysis by the proposed method and %R.S.D. was found to be less than 2%. The low %R.S.D. values indicated accuracy of the method.

Robustness

The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low %R.S.D. values indicated robustness of the method.

Ruggedness

The ruggedness of the proposed method was evaluated by two different analysts. The results for ABV, LMV and ZDV were found to be 99.85 %, 99.75 % and 100.01 %, 100.28 %, and 100.56, 100.45 respectively.

Repeatability

Repeatability of sample application was assessed by spotting (1000ng/spot) of drug solution seven times on a TLC, followed by development of plate and recording the peak area for seven spots. The % R.S.D. for peak area values of ABV, LMV and ZDV was found to be 0.89, 1.11 and 0.96, respectively.

The summary of validation parameters were listed in Table 2.

Table 2: Summary of validation parameter

Parameter	ABV	LMV	ZDV
Linearity range (ng spot ⁻¹)	500-4000	200-1200	300-1800
Correlation coefficient	0.9995	0.999	0.9994
Limit of detection (ng spot ⁻¹)	13.99	7.37	7.23
Limit of quantitation (ng spot ⁻¹)	42.4	22.32	15.23
% Recovery (n = 9)	0.69	0.8	0.8
Ruggedness (% R.S.D.)			
Analyst I	1.2	0.66	0.89
Analyst II	1.31	1.3	1.02
Precision (%R.S.D.)			
Repeatability of application (n = 6)	0.89	1.11	0.96
Inter-day (n = 3)	0.51-0.85	0.57-0.78	0.56-1.02
Intra-day (n = 3)	0.30-1.52	0.45-0.90	0.29-0.88
Robustness	Robust	Robust	Robust
Specificity	Specific	Specific	Specific

Table 3: Forced degradation of TDF and ETB

Sample exposure condition	Number of degradation products (R _f values)			Drug remained (1000 ng/spot)			Recovery (%)		
	ABV	LMV	ZDV	ABV	LMV	ZDV	ABV	LMV	ZDV
1M HCl, 8h, RT	2 (0.38, 0.56)	2 (0.26, 0.56)	2 (0.25, 0.38)	945.12	912.56	945.23	94.51	91.25	94.52
1M NaOH, 8h, RT	2 (0.29, 0.49)	2 (0.35, 0.61)	2 (0.18, 0.31)	939.23	922.45	941.52	93.92	92.24	94.15
10% H ₂ O ₂ , 8h, RT	1 (0.48)	2 (0.39, 0.51)	2 (0.21, 0.45)	963.56	953.26	956.23	96.35	95.32	95.62
Photo, 8 h	No degradation	No degradation	No degradation	996.89	992.85	1000.23	99.68	99.28	100.02
Heat, 3H, 55°C	No degradation	No degradation	No degradation	1000.23	998.12	1000.52	100.02	99.81	100.05

Stability- indicating property

The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure ABV, LMV and ZDV as well as some additional peaks at different R_f values. The spots of degraded

product were well resolved from the drug spot. The number of degradation product with their R_f values, content of ABV, LMV and ZDV remained, and percentage recovery were calculated and listed in Table 3.



CONCLUSION

The proposed HPTLC method provides simple, accurate and reproducible quantitative analysis for simultaneous determination of ABV, LMV and ZDV in tablets. The method was validated as per ICH guidelines.

Acknowledgements: The authors are thankful to Pravara Rural Education Society's College of Pharmacy (For Women), Chincholic, Nashik for providing necessary facilities. Authors are also thankful to Hetero labs for providing the gift sample of abacavir sulphate, lamivudine and zidovudine.

REFERENCES

1. WHO public assessment report, Abacavir Sulfate, Lamivudine and Zidovudine Tablets 300mg/150mg/300mg, 2009, Part 7, 1-2.
2. Indian Pharmacopoeia, Govt. of India, Ministry of Health & Family Welfare, Sector-3, RajNagar, Ghaziabed, 201002, vol. 2, 2007, 1276.
3. Indian Pharmacopoeia, Govt. of India, Ministry of Health & Family Welfare, Sector-3, RajNagar, Ghaziabed, vol. 2, 2007, 674.
4. The Merck Index, 13th Edn, Merck & Co., Inc., Whitehouse Station. NJ, 2001, 958.
5. Alagar RM, Amitrai SD, Determination of abacavir sulphate in tablet formulation and validation of simple spectrophotometric estimation in bulk and pharmaceutical formulation, *Int.J.Res.Pharm.Sci*, 2(2), 2011, 320-323.
6. Reddy RD, Theja DHH, Ruthu M, Sai BJNN, Reddy YP, Validated Spectrophotometric Method for Simultaneous estimation of Zidovudine and Lamivudine in Combined Pharmaceutical dosage form, *International Journal of PharmTech Research*, 4 (1), 2012, 311.
7. Sudha T, Saminathan J, Anusha K, Keerthi M, Bhargavi Y, Ganesan V, Simultaneous U.V. Spectrophotometric estimation of Lamivudine and Abacavir Sulphate in bulk and in tablet dosage form, *J. Chem. Pharm. Res.*, 2(5), 2010, 45-51.
8. Deepali G, Elvis M, UV Spectrophotometric Method for Assay of the Anti-Retroviral Agent Lamivudine in Active Pharmaceutical Ingredient and in its Tablet Formulation, *J Young Pharm.*, 2(4), 2010, 417-419.
9. Kumar P, Dwivedi SV, Khushnoor A, Validated HPTLC method for determination of Abacavir as bulk drug and in pharmaceutical dosage form, *Current Pharma research*, 1(4), 2011, 311-314.
10. Anbazhagan S, Indumathy N, Shanmugapandiyan P, Sridhar SK, Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets, *Journal of Pharmaceutical and Biomedical Analysis*, 39(3-4), 2005, 801-804.
11. Sudha T, Ravikumar VR, Hemalatha PV, Validated HPTLC method for simultaneous determination of lamivudine and abacavir sulphate in tablet dosage form, *International Journal of Pharmaceutical Sciences and research*, 1(11), 2010, 107-111.
12. Djurdjevic P, Laban A, Markovic S, Jelkic SM, Chemometric Optimization of a RP-HPLC Method for the Simultaneous Analysis of Abacavir, Lamivudine, and Zidovudine in Tablets, *Taylor & Francis Online : Analytical Letters*, 37(13), 2004, 2649-2667.
13. Mahor RP, Versha P, Singh Y, Sharma R, Bhandari A, Development and Validation of a HPLC Method for Simultaneous Estimation of Lamivudine and Zidovudine in Tablet Dosage Forms, *Der Chemica Sinica*, 2(6), 2011, 12.
14. Patro SK, Swain S R, Patro VJ, Choundhury NSK, Development and Validation of High Performance Liquid Chromatographic Method for Determination of Lamivudine from Pharmaceutical Preparation, *E-Journal of Chemistry*, 7(1), 2010, 117.
15. Manwar JV, Sonawane BV, Patil, SV, Takle SP, Rapid RP-HPLC Method for Estimation of Zidovudine from Tablet Dosage Form, *Der Chemica Sinica*, 2 (5), 2011, 152.
16. Balamuralikrishna K, Syama SB, Simultaneous determination of abacavir, lamivudine and zidovudine in tablet dosage forms by RP-HPLC method, *Journal of Pharmacy Research*, 4 (6), 2011, 1884.
17. Kenney KB, Wring SA, Carr RM, Wells GN, Dunn JA, Simultaneous determination of zidovudine and lamivudine in human serum using HPLC with tandem mass spectrometry, *J Pharm Biomed Anal*, 22(6), 2000, 967-983.
18. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for Human Use, Stability testing of new drugs substance and products Q1A (R2), 2003, 1-18.
19. Bakshi M, Singh S, Development of validated stability-indicating assay Methods—critical review, *J. Pharm. Biomed. Anal*, 28, 2002, 1011-1040.
20. Sethi PD, HPTLC: Quantitative Analysis of Pharmaceutical formulation, CBS Publications, New Delhi, 1996, 162-165.

Source of Support: Nil, Conflict of Interest: None.

