
# Method development and validation of RP- HPLC in the application of *in-vitro* dissolution study of Lamivudine in bulk drug and tablet formulation

**D.K.Mandloi[1]*, P.K.Tyagi[2], V.K.Rai[2], S. Dey[2], R.K Ashada[1], P.Mohanraj[1]**

[1]*Department of Pharmaceutical Sciences, Himalayan Pharmacy Institute, East Sikkim, India*
[2]*Shri RNS College of Pharmacy, Gormi, Bhind, M.P., India*

## Abstract

A new RP-HPLC method was developed for the determination of Lamivudine in the bulk drug and tablet dosage forms and it was applied for the *in-vitro* drug dissolution studies. Isocratic elution mode with a mixture of methanol and water in the ratio of (89:11) was selected as the mobile phase with a $C_{18}$ column (250 x 4.6mm, 5µ) for separation. This mixture was found to be appropriate allowing good elution for the Lamivudine at retention time 2.72 minute at flow rate of 1 ml/min and detection wavelength at 272nm. The linearity was found in the concentration range of Lamivudine 5-100 µg/ml. The liquid chromatography method was extensively validated for linearity, range, accuracy, precision (intermediate precision, repeatability) and specificity. All these analytical validation parameters were observed and the %RSD was determined which indicates the useful of RP-HPLC method for determination of Lamivudine in the bulk drug & tablets dosage form.

**Key words:** Lamivudine; Validation; *In-Vitro* studies; RP-HPLC

## Introduction

Nucleoside reverse transcriptase inhibitor (NRTIs) was the first class of drug those was introduced as antiretroviral agents for the treatment of infection with human immunodeficiency virus (HIV). Lamivudine (2'-deoxy-3'-thiacytidine: 3TC) is a pyrimidine analog reverse transcriptase inhibitor that is active against HIV-1, HIV-2, and HBV. The molecule has two chiral centers and is manufactured as the pure 2*R*, *cis* (-)-enantiomer. The racemic mixture from which lamivudine originates has antiretroviral activity but it has less potency and is substantially more toxic than the pure (-)-enantiomer. Compared with the (+)-enantiomer, the phosphorylated

(-)-enantiomer is more resistant to cleavage from nascent RNA/DNA duplexes by cellular 3'-5' exonucleases, which may contribute to its greater potency. [1]

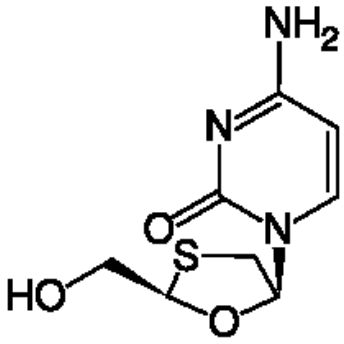


**Fig.1. Chemical structure of Lamuvidine**

There have been several publications describing analytical methods for the determination of lamuvidine [2-5]. Few methods have been described for the simultaneous determination other antiretroviral drugs in biological samples and pharmaceutical dosage forms [6-18]. Some of the earlier reported methods are having long analysis time (12 min [10], 17 min [17], 50 min [15], 25 min [18], 21 min [2], 30 min [16]) and some methods need liquid-liquid extraction of the sample before injection [19]. These are time consuming and costly methods. No method has been developed and applied to *in-vitro* pharmacokinetic studies for different classification of antiretroviral drug of Lamivudine by using single method.

Drug dissolution testing is an integral part of pharmaceutical development and routine quality control monitoring of drug release characteristics [20]. The profiles, obtained from dissolution rate studies, have also been used in an attempt to characterize the *in-vitro* behaviour of drug with success. They have to be performed under precisely specified conditions (i.e; temperature, volume, and stirring rate) that mimic process in the gastrointestinal tract.

The present work reports the development and validation of RP-HPLC for the determination of Lamivudine in bulk drug and tablet formulation and application of this method to *in-vitro* dissolution studies.

## Experimental Section

### *Chemicals*

Mobile phase consisted of methanol (SD Fine Chem. Ltd, HPLC grade) and water (Millipore HPLC grade) was used during analysis. For *in-vitro* dissolution studies was used. Lamivudine reference standard was generous gift from Cipla Pharmaceutical Ltd, Mumbai, India. Lamivudine tablets were procured from the local market lamivir-HBV 100 mg, lamivir 150mg (film coated tablets).

***Instrumentation*** Shimadzu LC-20AT liquid pump, SPD-20A UV-Visible detector, a Luna 5µ C-18 RP-HPLC column (250 x 4.6mm, 5 µm, ID), 25ml Hamilton injecting syringe and spinchrom

software were used. Sartorius (CP225D) electronic balance was used for weighing the materials. The dissolution rate studies of Limivudine from tablets were performed on a Veego, VDA-8DR USP dissolution apparatus

***Method development***

Various solvent systems were tried for the development of suitable HPLC method for the analysis of Lamivudine in the bulk and tablet formulation. The suitability of the solvent system was decided as the basis of the sensitivity of the assay, suitability for stability studies and availability of cost effective solvents.

***Preparation of stock solution***

Accurately weighed 100 mg of lamivudine standard was taken in separate 100 ml volumetric flasks and dissolved with 70 ml of mobile phase in each volumetric flask and sonicated for 5 min and then mark with mobile phase to get final concentration of 1mg/ml which was considered as stock solutions.

***Preparation of standards solution***

Standard stock solution was further diluted with mobile phase to obtain the concentration of solution in the range of 5-100 µg/ml was performed with UV detector for Lamivudine 272nm in 20µl of each prepared solutions were injected with a flow rate of 1ml/min and the peak area were recorded.

**Table.1 Calibration values of Lamivudine in standard drug**

| Concentration µg/ml | Peak area (mV.s)* |
|---|---|
| 5 | 123.881 |
| 10 | 269.736 |
| 15 | 371.494 |
| 20 | 503.710 |
| 30 | 748.583 |
| 40 | 983.960 |
| 50 | 1226.043 |
| 80 | 2006.804 |
| 100 | 2448.166 |

*Average of two determinations

The operation were carried out in triplicate group was plotted between concentration vs. peak area.

***Selection of analytical wavelength***

By appropriate dilution of standard stock solution with mobile phase, various concentration of Lamivudine was prepared accurately. The solutions were scanned between the wavelengths range 400-200 nm using the Shimadzu double beam UV visible spectrophotometer in the spectrum mode

***Preparation of sample solutions***

For the estimation of lamivudine from twenty tablets were weighed separately and powder equivalent to 100 mg of the drugs were accurately weighed and transferred to separate 100 ml volumetric flasks and sonicated for 5 minutes. Volumes were made up to the mark with mobile phase and filtered through 0.45µm filter. The concentrations of sample solution was prepared and analyzed with UV detection for Lamivudine at 272 nm, with a flow rate of 1ml/min.

***In Vitro Dissolution Studies from Tablet Dosage Form***

Drug dissolution studies were carried out in 900 ml of 0.1N HCl (pH 1.2, simulated gastric medium) according to the USP 2000 [21] dissolution procedures the single entity products with the use of a USP paddle type of apparatus at a stirring rate of 75 rpm. The temperature of the cell was maintained at 37 ± 0.5ºC. Aliquots of lamivudine were withdrawn at predetermined time intervals at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min replaced with equal volume of pre-warmed 0.1N HCL solution to maintain the sink condition. The sample solutions were diluted with methanol (HPLC grade) and it was analyzed by using methanol and water (89:11%, v/v) ratio and concentrations were determined at 272nm for Lamivudine peaks are given in Figure-3. The retention time of Lamivudine is 2.86 min. The cumulative percentage of drug released in the media was plotted against time in order to determine the release profile from the tablet formulations. The dissolution test data were obtained by averaging three parallel studies. (Table.2)

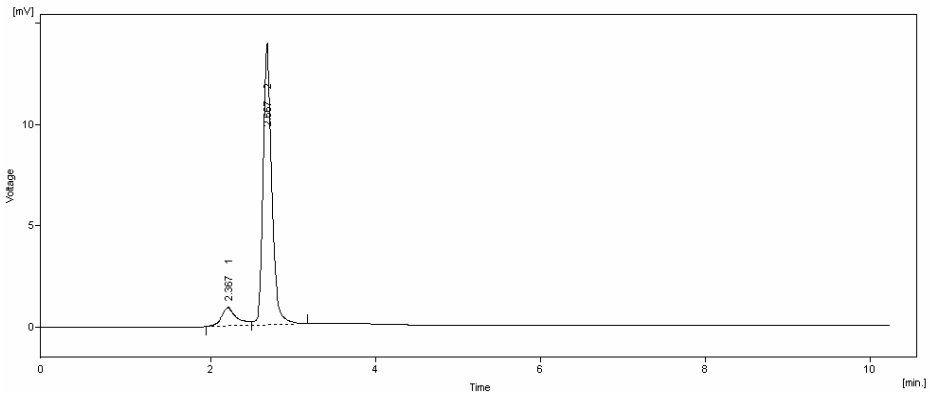


**Fig.3. RP-HPLC chromatograms Lamivudine by dissolution in-vitro kinetic studies**

**Table.2 Percentage cumulative release of Lamivudine**

| Time (min.) | Peak area[a] | Concentration (x) | Amount in 5ml | Amount in 900 ml | CR | %CR |
|---|---|---|---|---|---|---|
| 10 | 374.946[b] | 14.924[b] | 0.746 | 134.316 | 134.316 | 89.544 |
| 20 | 403.805 | 16.097 | 0.804 | 144.873 | 145.619 | 97.079 |
| 30 | 398.445 | 15.879 | 0.793 | 142.911 | 143.715 | 95.81 |
| 40 | 396.665 | 15.807 | 0.790 | 142.263 | 143.056 | 95.370 |
| 50 | 403.521 | 16.086 | 0.804 | 144.774 | 145.564 | 97.042 |
| 60 | 400.115 | 15.947 | 0.797 | 143.523 | 144.327 | 96.218 |
| 70 | 398.066 | 15.864 | 0.793 | 142.776 | 143.573 | 95.715 |
| 80 | 405.272 | 16.157 | 0.807 | 145.413 | 146.206 | 97.470 |
| 90 | 405.269 | 16.157 | 0.807 | 145.413 | 146.22 | 97.48 |
| 100 | 400.260 | 15.953 | 0.797 | 143.577 | 144.384 | 96.256 |
| 110 | 391.241 | 15.586 | 0.779 | 140.274 | 141.071 | 94.047 |
| 120 | 395.392 | 15.755 | 0.787 | 141.795 | 142.574 | 95.049 |

[a]n= Three times

[b]= at concentration and peak area mostly same as compared with developed method

## Results and Discussion

### *Method development:*

*Effect of incorporating methanol*

Methanol is a polar solvent being widely used in RP-HPLC. The primary effects it was found that the retention factor (k) was decreases with increases in the concentration of sample. The peak obtained was broad and showed severe tailing. However the final concentration of methanol was adjusted to achieve a good capacity factor. The chromatogram revealed that with increased in methanol concentration the retention time was reduced. The peak obtained was best acceptable at the methanol concentration of 89%. The peak was found sharp and reproducible

*Effect of changing the flow rate*

To determine the effect of flow rate, the programmed controller was set at different flow rates 0.5 mL/min, 0.7 mL/min, 0.9 mL/min 1.0 mL/min, 1.1 mL/min, 1.2 mL/min and 1.5 mL/min. operate were performed at each flow rate. The optimum flow rate was also chosen keeping in mind the recommended flow rate for a column with a given internal diameter.

***Method validation:***

*Linearity and range*

Under the optimized conditions, a calibration curve was used for Lamivudine. Six standard mixtures of different concentrations were prepared. The corrected peaks were used for constructing the calibration graph. Linearity range, regression equation, correlation coefficient, slope, standard errors of slope, standard errors of intercept, are shown in Table 2 The plot of peak area of each sample against respective concentration of Lamivudine was found to be linear in the range of 5-100 µg/ml, using regression analysis of the linear equation for Lamivudine Y= 24.60x + 7.205 with correlation coefficient of $r^2$= 0.9999. Linearity data obtained from the measurements are given in Table 3.

**Table.3 Data for the calibration graphs of Lamivudine**

| Parameters | Lamivudine |
|---|---|
| Linearity& range | 5-100µg/ml |
| Regression equation | y =24.60x +7.205 |
| Correlation coefficient | 0.9999 |
| Slope | 24.60 |
| Intercept | 7.205 |
| Standard error of slope | 0.0600 |
| Standard error of intercept | 3.525 |

***Accuracy***

In order to examine the accuracy of the method and to check the interference from excipients used in tablet dosage formulation, the recovery studies were carried out by standard addition method. In this method, three different amounts of Lamivudine were added to a constant known concentration of the composite tablet solution. Each solution was injected five times and the amounts determined were compared to theoretical amounts. The results were summarized in Table 4. The recovery range was found from 99.01 to 99.69 for Lamivudine.The relative standard deviation (% RSD) of Lamivudine 1.42 was observed which indicates that the method gives sufficient accuracy.

**Table. 4: Table for accuracy of the result**

| Sample | Label claim (mg) | Fortified amount (mg) | Amount[a] found (mg) | Recovery (%) | % R.S.D |
|---|---|---|---|---|---|
| Lamivir | 150 | 10 | 9.93 | 99.01 | 1.05 |
| | | 20 | 19.16 | 99.22 | 1.89 |
| | | 30 | 29.04 | 99.69 | 1.42 |

[a]n= Five times

***Precision***

Precision of the method was tested by performing intra-day and inter-day studies. For intra-day studies, triplicate of prepared samples were analyzed within same day. For inter-day validation, concentrations were determined on three separate days. The % RSD values obtained from peak area for LAM was observed. It can be seen from Table 5, a good precision was obtained for drug.

**Table 5 Intra-and inter-day precision**

| Analyte | Concentration (µg/ml) | Intra-Day | %RSD[a] (n= 3) | Inter- Day | %RSD[b] (n= 9) |
|---|---|---|---|---|---|
| LAM | 20 | 493.104 | 0.907 | 504.424 | 1.052 |
| | 40 | 970.377 | 0.513 | 986.628 | 0.545 |
| | 80 | 1973.850 | 0.879 | 1960.273 | 0.815 |

[a]n=Three times
[b]n=Three days

***Limit of Detection (LOD) and Limit of Quantification (LOQ)***

The limit of detection (LOD) and limit of quantification (LOQ) for Lamivudine was found to be 1.705, 5.16 µg/ml. The typical chromatogram of Lamivudine was shown in Figure 2. A mixture of methanol and water in the ratio of (89:11, v/v) was found to be most suitable to obtain a peak well defined and free from tailing.

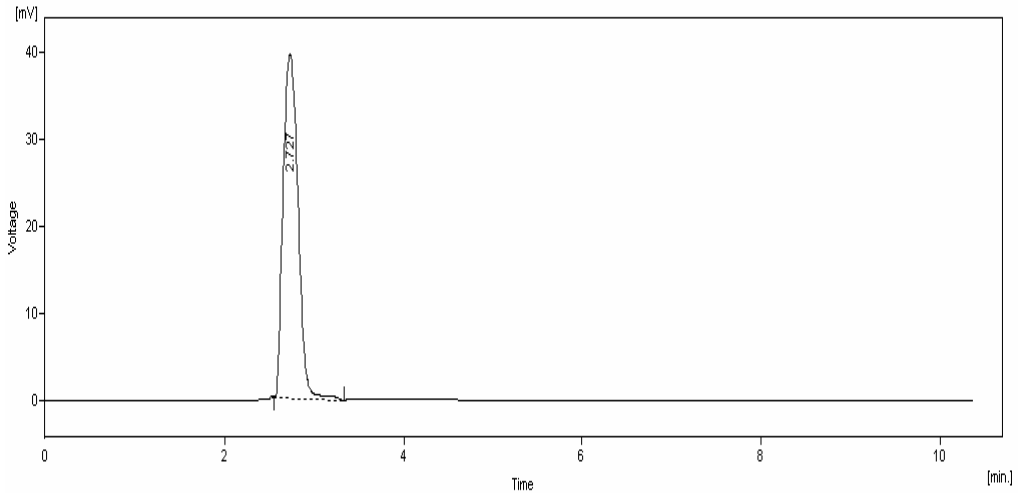


**Fig. 2. RP-HPLC chromatogram Lamivudine**

***Specificity***

The effect of inactive ingredients (placebo) on the determination of the drug in tablet formulation was studied. Each tablet contains 150mg of LAM along with some inactive ingredients, such as titanium dioxide, red oxide, yellow oxide, talc and methyl cellulose. In the chromatogram, no interfering peaks were observed in the region of analytes.

***Robustness***

Robustness of the method was tested by small but deliberate variations of flow rate, mobile phase and temperature. Effects of variation in the flow rate (±1 ml/min) were studied at three different concentrations and temperatures (±1˚C to ±5˚C) were studied. Effect of variation in the different mobile phase ratio also studied at three different concentrations.

***Ruggedness***

A different analyst, using a different batch of chemicals, column, tried the method. There was not much variation in results calculated by % RSD. These studies showed that the method is rugged.

***System suitability parameter***

The system suitability test was carried out on freshly prepared standard stock solution of Lamivudine. Parameters such as efficiency, resolution, peak tailing HETP, LOD, and LOQ that were studied to evaluate the suitability of the system are given in Table 6.

**Table.6 System suitability Test for Lamivudine**

| Parameters | LAM |
|---|---|
| Retention Time | 2.7 |
| HETP(mm) | 0.022 |
| Tailing factor | 1.12 |
| Theoretical plates/m | 32247.057 |
| LOD(µg/mL) | 1.705 |
| LOQ(µg/mL) | 5.16 |

***Applied to the dissolution studies:***

The method was also applied to determine the release rate pattern of the drug from the tablet dosage form of Lamivudine in dissolution rate studies. The release data were evaluated according to different models namely Zero order, First order, Higuchi and Korsmeyer& Peppas function. The regression coefficient values obtained after fitting the dissolution data to various kinetic models is shown in Table 7. The regression value of Lamivudine was found to be following the Korsmeyer-Peppas release pattern following diffusion controlled release of the contained drug.

The assay of Lamivudine tablet was found to be 149.39, & 99.83. The results are given in Table 8 The absence of additional peaks in the chromatogram indicates non interference of the common excipients used in the tablets. Lamivudine was found to give individual identical results when compared to their peak area and peak height when samples were collected and analyzed by developed RP-HPLC method using the same solvent system. Drug release kinetics was determined by analyzing the regression values.

**Table. 7: Kinetic assessments of release data**

| Kinetics | Parameter | LAM |
|---|---|---|
| Zero order | $Kr^0$<br>$r^2$ | 0.328<br>0.231 |
| First order | $Kr^1$<br>$r^2$ | 0.001<br>0.044 |
| Highuchi | $Kr^2$<br>$r^2$ | 0.122<br>0.021 |
| Korsmeyer&Peppas | $Kr^3$<br>$r^2$ | 0.350<br>0.705 |

$Kr^0$, $Kr^1$, $Kr^2$, $Kr^3$=slope values, $r^2$=regression values

The present study indicates the possibility of using the same solvent system for quality control samples and for analyzing the drug release pattern from the tablet dosage forms.

**Table. 8: Results of Lamivudine analysis in tablet dosage forms**

| Sample | Label claim (mg) | Amount found | %Recovery[a] | % RSD |
|---|---|---|---|---|
| Tablet-A<br>Lamivir | 150 | 149.39 | 99.59 | 0.730 |
| Tablet-B<br>Lamivir-HBV | 100 | 99.83 | 99.83 | 0.669 |

[a]n= Five times

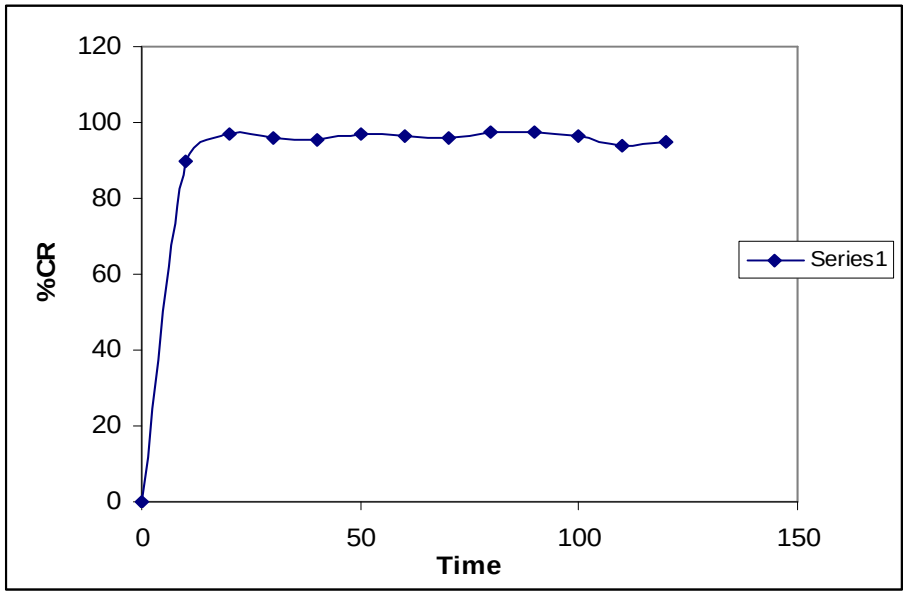


**Fig.4: *In-vitro* dissolution profile of Lamivudine**

## Conclusion

Developed reverse phase high performance liquid chromatographic method is simple, reliable, linear, accurate, sensitive and reproducible as well as economical for the effective qualitative and

quantitative analysis of Lamivudine in bulk drug and tablet formulations. The method does not involve any costly sample extraction procedure in the method which highlights its usefulness in routine quality control testing. In addition, the reported method can also be used for pharmacokinetic studies.

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