Development and validation of analytical method for the estimation of lamivudine in rabbit plasma

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Abstract Lamivudine has been widely used in the treatment of HIV disease. A reliable, sensitive reversed phase high performance liquid chromatography (RP-HPLC) method was developed and validated for lamivudine in rabbit plasma. The method was developed on Hypersil BDS C-18 column (250 mm × 4.6 mm, 5 μm) using a mobile phase of 0.25% Triethylamine buffer (pH 3.0): acetonitrile (70:30, v/v). The efficient was monitored by UV detector at 256 nm. The total run time was 15 min with a flow rate of 1.0 mL/min. Calibration curve was linear over the concentration range of 25–2000 ng/mL. The retention times of lamivudine and internal standard (Nelfinavir) were 8.78 min and 10.86 min, respectively. The developed RP-HPLC method can be successfully applied for the quantitative pharmacokinetic parameters determination of lamivudine in rabbit model.

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1. Introduction
Beside global commitment to eradicate the HIV/AIDS, this disease has spread significantly in recent years. Nearly 3.0 million deaths have occurred due to this pandemic disease each year. In recent years a new ART (antiretroviral) therapy has been developed, which includes single or combination therapy by antiviral drug. Lamivudine is an analogue of cytidine. It inhibits both types (I and II) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3′-OH group in the incorporated nucleoside analogue prevents the formation of the 5′-3′ phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated [1]. Few methods have been developed for lamivudine determination by RP-HPLC in human plasma [2–6]. Reviewing the literature revealed that neither RP-HPLC nor any other chromatographic method reported for the determination of this drug in rabbit plasma [7].

This paper describes the development and validation of a RP-HPLC assay, using UV detector for the quantitative determination of lamivudine in rabbit plasma.
2. Materials and methods

Lamivudine was the generous gift from Ranbaxy Pharmaceuticals Limited, Paonta Sahib, India. Lamivudine (Cipla, India) was purchased from local market. HPLC grade acetonitrile was procured from Qualigens, India. Potassium dihydrogen phosphate (KH₂PO₄) and ortho-phosphoric acid were purchased from Loba Chemie, Mumbai, India. All other chemicals and solvents used were of analytical grade. Water used in the HPLC analysis was prepared by the water purifier (Arium®, 611UF, Sartorius, Germany). The mobile phase and all the solutions were filtered through a 0.45 μm Ultipor® N66® membrane filter (Pall Life Sciences, USA) prior to use.

2.1. Instrument and software

HPLC system (Waters, USA) consisting of quaternary pump (Watrex™600), 7725i rheodyne manual injector and UV–Vis detector of module 2998 and empower-II software were used for analysis. The plasma samples were processed by using of Micropipettes (Ependruff, USA), Spinix Vortexer (M37610-33, Barnstead international, USA), Biofuge Fresco Centrifuge (Heraeus, Germany), Ultra-Sonicator (Loba Chem, Mumbai), Spinix Vortexer (M37610-33, Barnstead international, USA). The plasma samples were then centrifuged (Biofuge Fresco Centrifuge, Heraeus, Germany) at 10,000 rpm for 5 min at 4 °C and finally organic solvent was evaporated under nitrogen atmosphere. The samples were then reconstituted by addition of 100 μL of diluents. The samples were then vortexed using multi-pulse vortexer and finally 20 μL sample volume was injected into HPLC system for analysis.

2.2. Preparation of stock solution of lamivudine

An accurately weighed 10 mg of lamivudine was dissolved in 10 mL of methanol and transferred into a 50 mL volumetric flask. The stock solution (1000 μg/mL) was stored in a refrigerator (2–4 °C). From the stock solution working solutions were prepared by serial dilution.

2.3. Preparation of internal standard (IS) stock solution

An accurately weighed nelfinavir (10 mg) was dissolved in 10 mL of methanol to obtain 1000 μg/mL of stock solution. The stock solution was diluted with methanol to obtain a solution of 50 μg/mL of nelfinavir.

2.4. Extraction efficiency

The recovery of an analyte is the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Different organic extraction solvents (ethyl acetate, dichloromethane, acetonitrile, methyl alcohol, hexane and chloroform) were tried in the experiment, and acetonitrile was proved to be the most efficient in extracting lamivudine from rabbit plasma and had a small variation in extraction recoveries over the concentration range. Spiked plasma sample was prepared in triplicate at single concentration of 400 ng/mL of lamivudine and 50 μg/mL of IS, and assayed as described above. The extraction efficiency of lamivudine was determined by comparing the peak areas measured after analysis of spiked plasma sample with those found after direct injection of non-biological (unextracted) samples into the chromatographic system at the same concentration levels.

2.5. Preparation of plasma samples

Plasma samples for calibration and quality control analysis were prepared by mixing of 100 μL of sample and 10 μL of IS solution (50 μg/mL). The mixture was vortexed (Glas-COL, USA) for 10 s and then 1.5 mL of CAN was added and finally the samples were extracted for 10 min in a spinix vortexer (M37610-33, Barnstead international, USA). The plasma samples were then centrifuged (Biofuge Fresco Centrifuge, Heraeus, Germany) at 10,000 rpm for 5 min at 4 °C and finally organic solvent was evaporated under nitrogen atmosphere. The samples were then reconstituted by addition of 100 μL of diluents. The samples were then vortexed using multi-pulse vortexer and finally 20 μL sample volume was injected into HPLC system for analysis.

2.6. Preparation of calibration curve

Standards were prepared in fresh medium by spiking 20 μL each of the working solution of lamivudine into the control blank plasma (180 μL) to give the concentration range (25.86, 50.77, 101.53, 203.06, 406.12, 846.09, 1244.24, 1637.16 and 2046.45 ng/mL).

2.7. Validation of methods

The validation of an analytical method confirms the characteristics of the method to satisfy the requirements of the application. The method was validated following ICH guidelines for specificity, recovery, linearity, precision and stability. Under he validation study the following parameters were studied:

2.7.1. Specificity

The specificity criterion demonstrates that the results of the method are not affected by the presence of interference, i.e. whether the compound of interest elutes without interfering with other compounds and components of plasma. The specificity of the method was determined by comparing the chromatograms obtained from the aqueous samples of lamivudine and IS with those obtained from blank plasma. Blank plasma samples from each of three rabbits were processed in presence of IS. The solutions each containing 404.16 ng/mL were injected into the column under the optimized chromatographic conditions to obtain the chromatographic peaks of lamivudine in plasma in presence of IS.

2.7.2. Linearity

Quantitative analytical results are highly influenced by the quality of the calibration curve. Nine different concentrations of lamivudine with fixed concentration of IS in blank plasma were processed and calibration curve was constructed in the specific concentration range. The calibration curve was plotted between the ratio of peak areas of lamivudine to IS and concentration of lamivudine.

2.7.3. Precision and accuracy

Both repeatability (within a day precision) and reproducibility (between days precision) were determined. Three quality control samples were subjected for the study. Five injections of each of the specified quality control samples at three levels
2.7.4. Stability
The quality control standards containing 204.16, 402.23 and 801.69 ng/mL of lamivudine were subjected for detection of stability of the drug in plasma. One set of six samples each was kept in polypropylene tube and subjected to freeze-thaw cycles each at −20°C and for 24 h. The second sets of six samples each was kept at room temperature for 1 month. All the samples were analyzed by standard chromatographic conditioned to determine their peak areas. Samples were considered to be stable when the final assay values of samples were found similar to that of the initial assay value of the drug.

2.8. Pharmacokinetic study in rabbits
The method described above was applied to quantify the plasma concentration of lamivudine in a single-dose pharmacokinetic study conducted on three white male albino rabbits. The protocol was approved by the Institutional Ethical Committee at the Gayatri College of Pharmacy, Orissa, India. The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals) guidelines. The rabbits weighing 1.5–2.5 kg were housed with free access to food and water, except for the final 12 h before experimentation. After a single oral administration of 100 mg of lamivudine (LamivirTM), 2.0 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h time-points into heparinized collection tubes. The blood was immediately centrifuged (1000 × g) for 10 min at an ambient temperature. The supernatant plasma layer was separated and stored at −20°C until analyzed. The plasma samples were analyzed for lamivudine concentrations as described above. The total area under the observed plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule. The first order elimination rate constant (K_e) was estimated by the least square regression of the points describing the terminal log–linear decaying phase. \( T_{1/2} \) was derived from \( K_e \left( \frac{T_{1/2}}{2} = \ln 2 / K_e \right) \). The absorption rate constant (K_a) was determined by residual method. The maximum observed lamivudine concentration (C_max) and the time at which C_max was observed (T_max) were reported directly from the profile.

3. Result and discussion
Normal phase chromatography can be used for the separation of non-ionic and non-polar substances, while reversed phase chromatography (C_8 and C_18 column) can be used for the separation of non-ionic, ionic non-polar and semi polar substances. Octadecylsilane (C_18 column) reversed phase column was selected for lamivudine. A mixture of 0.25% Triethylamine buffer (pH 3.0) and acetonitrile was used as mobile phase for the analysis of lamivudine. The optimum ratio of buffer and acetonitrile used in the current investigation was 70:30 (v/v), which was selected on the basis of resolution and absence of interference. The best resolution and sensitivity of the method was obtained at 256 nm with 1 mL/min flow rate of the mobile phase.

3.1. Specificity
Typical chromatogram (Fig. 1) of mixture of lamivudine and IS revealed that they are well separated under HPLC condition applied. A chromatogram of blank plasma sample is shown in Fig. 2. The retention time was found to be 8.787 min for lamivudine and 10.864 min for IS. Fig. 3, which is the overlapping of the chromatogram of lamivudine (Fig. 1) with the chromatogram of blank plasma (Fig. 2), indicated absence of interference of plasma components around the zone of retention time of lamivudine analysis. The chromatograph of medium level quality control sample i.e. 408.19 ng/mL of lamivudine (Fig. 4) showed a good resolution peaks for
Figure 2  Chromatogram of blank plasma of rabbit.

Figure 3  Overlapping of chromatograms of lamivudine–IS mixture with blank plasma.

Figure 4  Chromatogram of medium level quality control sample of lamivudine.
lamivudine and IS which are well differentiated from the peaks of plasma components.

3.2. Selection of extraction solvents

The solvent which was capable of extracting maximum amount of drug from plasma sample was selected for processing of the samples. The recovery of drug was found to be 83.52% ± 2.1, 76.46% ± 1.7, 71.08% ± 2.2, 62.88% ± 1.1 and 59.04% ± 0.8 (w/w) in acetonitrile, hexane, chloroform, dichloromethane and methanol respectively at all three concentration levels, which confirm the extraction efficiency of the solvents. Among the solvents, acetonitrile showed maximum amount of drug recovered. Hence, acetonitrile was selected as the extracting solvent. The recovery of IS was also found to be maximum in acetonitrile which was 91.19% ± 2.2 (w/w).

3.3. Linearity range

The ratio of peak area of lamivudine to IS at various concentrations of lamivudine in plasma is shown in Table 1. A calibration curve was plotted between peak area ratios of lamivudine to IS versus lamivudine concentration in plasma, which is presented in Fig. 5. The chromatographic responses (ratio of peak area of lamivudine to IS versus lamivudine concentration) were found to be linear over an analytical range of 25.38–2046.45 ng/mL with regression coefficient value of 0.998, which showed reproducibility. The regression equation value of the calibration curve is presented in Table 2.

3.4. Precision and accuracy

The accuracy of the measurements was determined by using three quality control samples and the results are presented in Table 3. The relative standard deviation (RSD) of intra-day assay of the drug was ranged from 3.3 to 8.4% and for the inter-day assay was from 4.7 to 10.3%. Accurate data ranged from 98.52 to 99.97% for both the conditions indicated that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of lamivudine resulted a RSD value less than 10.3% (should be less than 15% according to CDER guidance for bioanalytical method validation), which revealed that the precision of the proposed method is very high.

3.5. Stability

The result of the stability validation is presented in Table 4. The results revealed that the final concentration of the drug in each quality control samples at stability conditions i.e. freeze–thaw condition, 24 h storage and one month storage was found to be similar with initial concentration. The RSD value (n=6) of final concentration of drug after storing the samples in all the stability conditions was found to be less than 15%. The accuracy of stored samples was found to be nearly equivalent to 100%. Hence, it can be inferred that lamivudine was stable in rabbit plasma.

3.6. Pharmacokinetic study in rabbits

The developed method was applied to quantify lamivudine concentration in pharmacokinetic study carried out on rabbits. The mean plasma concentration versus time profile following a single oral administration of lamivudine to three rabbits is presented in Fig. 6. Various other pharmacokinetic parameters have been summarized in Table 5.

4. Conclusion

A novel, simple and sensitive RP-HPLC method has been developed and validated for the estimation of lamivudine in rabbit plasma using UV-detector. A good resolution was obtained between lamivudine and IS with retention time of
8.787 and 10.864 min, respectively. There was no interference i.e. peaks observed around the retention time of lamivudine and IS. The method was found to be linear ($R^2 = 0.998$) within the analytical range of 25.54–2040.75 ng/mL. A maximum recovery of drug from plasma was resulted using acetonitrile as extracting solvent in comparisons to other organic solvents.

The results obtained proved that the RP-HPLC method development was accurate and reproducible and the drug was stable in rabbit plasma. In summary, the optimized chromatographic estimation of lamivudine with good resolution can be used for evaluating the bioavailability and also applied to routine therapeutic monitoring of the lamivudine.

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