

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE BY RP-HPLC METHOD

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ABSTRACT: A simple, accurate, precise and sensitive HPLC method with UV detection was developed and validated to separate and detect lamivudine in human plasma using Stavudine as an internal standard. Lamivudine (3-TC) and Stavudine (internal standard) were extracted from human plasma using methanol protein precipitation and were chromatographed on a Phenomenex C18 (250X4.6mm., 5 μ m particle size) column using 20 μ l injection volume and detection at 270nm. An isocratic mobile phase consisting of Methanol: Water (85:15%v/v) was used to separate these drugs. The retention times of lamivudine and I.S were 4.6 and 6.2 respectively. The method was validated over the range of 406.10-4020.05 ng/ml. The limit of detection was 200 ng/ml and the limit of quantification was 400 ng/ml for 3-TC. Within and between-day precisions are less than 6.5% for all quality control samples. The absolute recoveries of 3-TC was greater than 90% were achieved. The described method can be readily utilized for analysis of pharmaceutical products.

Keywords : Lamivudine ; 3-TC ; Stavudine ; HPLC.

1. INTRODUCTION

Lamivudine was initially developed for the treatment of HIV infection. Lamivudine the (-) enantiomer of 2'-deoxy-3'-thiacytidine, is a nucleoside analog in which the 3' carbon of the ribose of zalcitabine has been replaced by sulfur. The (-) enantiomer of the racemic mixture shows much less cytotoxicity than the positive enantiomer (3). Although generally less potent than zidovudine or zalcitabine in inhibiting HIV-1 and HIV-2 replication in vitro, lamivudine has very low cellular cytotoxicity. It causes competitive inhibition of reverse transcriptase activity with respect to dCTP. Following oral administration, lamivudine is rapidly absorbed with bioavailability of approximately 80%. Lamivudine also shows inhibitory activity against HBV in vitro and chronically infected human beings. The chemical structure of lamivudine was shown below (Fig.1).

Lamivudine (3-TC), 2'-deoxy-3'-thiacytidine, is less potent than zidovudine and zalcitabine, but an antiviral regimen that includes 3-TC is desirable, since 3-TC had been shown to be somewhat less toxic than other NRTIs (6). Moreover, lamivudine is active against zidovudine-resistant HIV (4). The US Department of Health and Human Services' current guideline for the treatment of established HIV infection strongly recommends 3-TC in combination with another NRTI and either a protease inhibitor or efavirenz (12). The mechanism of action of lamivudine seems to be similar to that of zidovudine (9). 3-TC has approximately 80% oral bioavailability in human and its usual dosage is 150 mg twice daily or 300 mg once daily in combination with other antiretroviral agents (5). In such doses, the compound is well tolerated (4). Several high-performance liquid chromatographic (HPLC) methods with ultraviolet detection for the determination of 3-TC in biological fluids have been

published. (7) used protein precipitation with trichloroacetic acid for sample cleanup for 3-TC analysis in human serum. Solid-phase extraction (SPE) methods have been developed by different authors (8, 1, 2). (11) incorporated a column switching scheme into the procedure for measuring 3-TC in urine. Other methods for 3-TC quantification include radioimmunoassay and mass spectrometry (5).

This paper describes the development and validation of a sensitive, specific, rapid, simple and economic HPLC bioanalytical method for 3-TC quantification in human plasma. This report represents a one-step sample preparation using methanol that simplifies the analysis of lamivudine in human plasma. Sample handling and chromatographic run times were minimized to provide fast quantitative results while maintaining the specificity accuracy and precision required for evaluation of lamivudine.

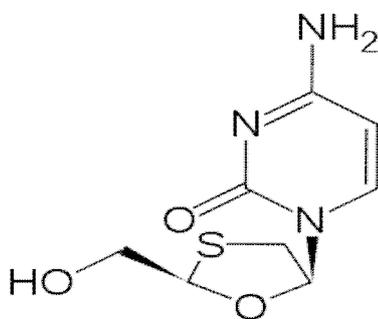


Fig. 1 Structure of Lamivudine

2. EXPERIMENTAL

2.1. Chemicals and Reagents

Working standards of lamivudine (3-TC) and Stavudine (I.S) were kindly provided by Micro Labs Pvt Ltd., Bangalore. HPLC Grade solvents (Acetonitrile, water and methanol) were obtained from S.D.Fine chemicals Ltd., India, Ranbaxy India Ltd., Qualigens Fine Chemicals Ltd., Mumbai.

Stock standard solutions of lamivudine and the internal standard were prepared by dissolving appropriate amounts of compounds in a known volume of methanol and water stored at 4°C (10).

Blank human blood was collected with heparin from healthy and drug free volunteers. After centrifugation at 5000 rpm at room temperature, plasma was collected and stored at -30°C until analysis.

2.2. Instrumentation

Analyses were performed on Shimadzu scientific instruments composed of LC-20 AT pump and SPD- 20 AT variable wavelength detector. The separation of compounds was achieved using a Phenomenex Luna C18 column (5µm, 25cm X 4.6mm i.d).

2.3. Chromatographic condition [10]

The mobile phase used was methanol and water (85:15% v/v). Before analyses, the mobile phase was filtered through 0.4 µm filter and then degassed ultrasonically for 15 min. The analyses were conducted at a flow rate of 1.0 ml/min. The eluent was monitored at a wavelength of 270nm for lamivudine. The total run time was 10 min.

2.4. Protein Precipitation [6]

The blank plasma sample was prepared by adding 1ml of plasma and 1ml of methanol and vortex for 30sec. Then centrifuge the solution at 4°C, 5000 RPM for 5min. The supernatant liquid is taken and transferred to HPLC vials

The blank plasma sample was prepared by adding 1ml of spiked plasma 1ml of methanol and 0.2ml of IS std(250µg/ml) and vortex for 30sec. then centrifuge the solution at 4°C, 5000 RPM for 5min. the supernatant liquid is taken and transferred to HPLC vials.

2.5. Quantification of 3-TC in plasma

A Standard curve was prepared by injecting various concentrations of lamivudine in plasma. The concentrations of the plasma and quality control samples were calculated by using the regressed equation of the straight line $y=mx+c$.

The limit of detection (LOD) and limit of quantification (LOQ) were determined as follows: 6 blank samples from six separate subjects for each analyte and matrix were extracted and compared to a low standard of each analyte. Where an obvious peak existed at the same retention time as the analyte, a concentration was calculated for this peak. Where no discreet peak or a series of small noise peaks existed at the same retention time as the analyte, the height of the noise was measured and compared to the height of the low standard. This provided a "concentration" for the noise. An average of the 6 "noise concentrations" was calculated and multiply by either 3 (LOD) or 5 (LOQ). LOQ values were subsequently confirmed using six replicates spiked at the target concentration as being within an acceptable variance of 20%.

2.6. Determination of accuracy, precision and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying two replicate samples of plasma at 3 different lamivudine concentration(3420.06, 2052.04 and 1026.02 ng/ml) in 6 analytical runs. Accuracy was measured as the percent deviation from the nominal concentration.

The absolute recovery of lamivudine and I.S were determined by comparison of the peak areas from non-extracted and extracted samples of QC-3 in triplicate. The recovery was calculated as the relative standard deviation of the mean (R.S.D.) with R.S.D. (%) = (standard deviation of the mean/mean) ×100.

2.7. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of blank human plasma collected from 6 drug-free volunteers. Chromatograms of plasma from all drug-free volunteers were examined that may co-elute with 3-TC.

2.8. Stability studies

The stability of solutions of 3-TC was assessed in analytical standard solutions, processed sample extracts and biological matrix by comparison to freshly spiked plasma samples.

2.8.1. Stability of analytical standard solutions

The stability of solutions of 3TC and Stavudine in methanol and water (85: 15 % v/v) stored at 10°C was examined for 6 months. Standard solutions of 3-TC and Stavudine were prepared throughout the method development phase of the study and stored at 10°C. These solutions were compared to freshly prepared standards of 3-TC and Stavudine. Standard solutions prepared in mobile phase and stored at 10°C were stable for at least 6 months.

2.8.2. Short term stock stability

A Stock solution of lamivudine and IS was kept at room temperature for 8 hours.

2.8.3. Long term stock stability

A Stock solution of lamivudine and IS was kept at room temperature for 45 days.

2.8.4. Bench top stability

The replicate concentration of low and high quality control samples were determined by comparing the mean area ratio of freshly thawed samples which kept at room temperature for about 6 hours.

2.8.5. Coolant stability

The replicate concentration of low and high quality control samples were determined by comparing the mean area ratio of freshly thawed samples which kept at room temperature for about 24 hours.

2.8.6. Freeze thaw stability

The stability of low and high quality control samples was determined after three freeze thaw cycles. The percent degradation was determined by comparing the mean of area ratio of lamivudine.

2.8.7. Long term plasma stability

At least three aliquots of each of low and high concentrations at same conditions as study samples. Analyse on three separate occasions. Storage time should exceed the time between the date of first sample collection and the date of last sample analysis.

2.9. Ruggedness

This includes different analysts, laboratories, columns, instruments, sources of reagents, chemicals, solvents.

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test condition. The ruggedness of the method was studied by changing the experimental condition such as,

- Changing to another column of similar type (Phenomenex Gemini C18)
- Different operation in the same laboratory.

3. RESULTS AND DISCUSSION

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability. (Table 1)

3.1. Linearity

The method was validated over the range of 406.20–4020.05 ng/ml. The slope, correlation coefficient were found to be 86.139, 0.9999 respectively.

3.2. Sensitivity

In the plasma, the calculated limit of quantification was 400ng/ml and limit of detection was 200ng/ml for lamivudine.

3.3. Recovery

The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery of the drug and the internal standard was calculated and it was 97.55% and 98.11% respectively.

3.4. Precision and Accuracy

The accuracy, precision and intraday precision were carried out by preparing 6 individual samples of HQC, MQC and LQC and the percentage C.V. and percentage nominal was calculated.

3.5. Stability

Stability of the method was carried out by performing short term and long term stock stability. The percentage mean ratio of the drug and internal standard were calculated.

Stability of the plasma samples was carried out by performing coolant, bench top and freeze thaw stability. The percentage mean ratio of the HQC and LQC were calculated.

The long term plasma stability was carried out by performing from the initial sample to the date of last sample. The percentage CV of the HQC and LQC were calculated.

3.6. Ruggedness

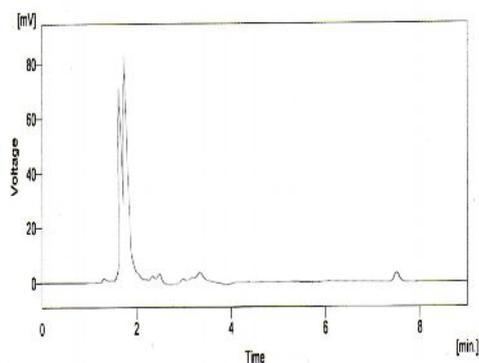
The ruggedness of the method was carried out by changing by column and by different analyst in the same lab. The percentage CV of the HQC and LQC were calculated.

4. CONCLUSION

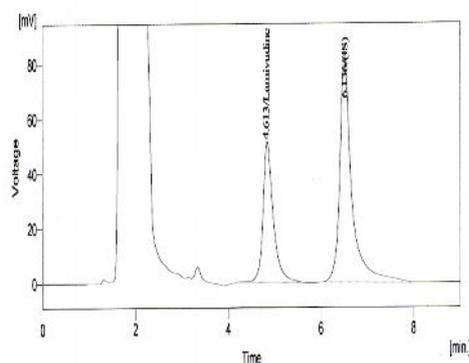
The bioanalytical method developed is simple and shows good accuracy, specificity and reproducible. It can be used for the estimation of Lamivudine in biofluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of lamivudine without any interference. The developed method could be applied in bioequivalence, pharmacokinetic and toxicokinetic studies.

TABLE: 1

S.NO	PARAMETERS	RESULT
01.	System suitability	Pass
02.	Selectivity	Pass
03.	LOD	200 ng
04.	LOQ	400 ng
05.	Accuracy and precision	Pass
06.	Linearity	$R^2 = 0.9999$
07.	Short term stock stability	12 hrs
08.	Long term stock stability	30 days
09.	Coolant stability	7 days
10.	Bench top stability	12 hrs
11.	Freeze thaw stability	Pass (3 cycles)
12.	Long term plasma stability	60 days
13.	Ruggedness	Pass



(a)



(b)

Fig. 2. Typical chromatogram of (a) Blank human plasma, (b) Blank human plasma spiked with 3-TC and Stavudine

REFERENCES

- Physicians Desk Reference (PDR), 54th Ed.; Medical Economics Company Inc.: Montvale, NJ, 2000; 1172, 2000.
- Goodman & Gilman's, The Pharmacological Basis of Therapeutics, (CDROM) 9th Ed.; Hardman, J.G., Limbird, L.E., Eds.; McGraw-Hill Companies: Hightstown, NJ, 1996.
- Alnouti, Y., White, C.A., Bartlett, M.G., 2004. Determination of lamivudine in plasma, amniotic fluid, and rat tissues by liquid chromatography. *J. Chromatogr. B* 803, 279–284.
- Balint, G.A., 2001. Antiretroviral therapeutics possibilities for human immunodeficiency virus/acquired immunodeficiency syndrome. *Pharmacol. Ther.* 89, 17–27.
- Eunice Kazue Kano, Cristina Helena dos Reis Serra., 2005. Determination of lamivudine in human plasma by HPLC and its use in bioequivalence studies. *International Journal of Pharmaceutics*. 297, 73–79.
- Joanna J. Zheng, Steven T. Wu., 2001. High-performance liquid chromatographic assay for the determination of 2'-deoxy-3'-thiacytidine (lamivudine) in human plasma. *Journal of Chromatography B*. 761, 195–201

7. Estrela, R.C., Salvadori, M.C., Suarez-Kurtz, G., 2004. A rapid and sensitive method for simultaneous determination of lamivudine and zidovudine in human serum by on-line-phase extraction coupled to liquid chromatography /tandem mass spectrometry detection. *Rapid Commun. Mass Spectrom.* 18, 1147–1155.
8. Harker, A.J., Evans, G.L., Hawley, A.E., Morris, D.M., 1994. High-performance liquid chromatographic assay for 2'-deoxy- 3'-thiacytidine in human serum. *J. Chromatogr. B* 657, 227– 232.
9. Hart, G.J., Orr, D.C., Penn, C.R., Figueiredo, H.T., Gray, N.M., Boehme, R.E., Cameron, J.M., 1992. Effects of (-)-2'-deoxy-3'-thiacytidine (3-TC)-5-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerase alpha, beta and gamma. *Antimicrob. Agents Chemother.* 36, 1688–1694.
10. Hoetelmans, R.M.W., Profijt, M., Meenhorst, P.L., Mulder, J.W., Beijnen, J.H., 1998. Quantitative determination of (-)-2'-deoxy-3'-thiacytidine (lamivudine) in human plasma, saliva and cerebrospinal fluid by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B* 713, 387–394.
11. Morris, D.M., Selinger, K., 1994. Determination of 2'-deoxy-3'- thiacytidine (3TC) in human urine by liquid chromatography: direct injection with column switching. *J. Pharm. Biomed. Anal.* 12, 255–264.
12. Pereira, A.S., Tidwell, R.R., 2001. Separation methods for nucleoside analogues used for treatment for HIV-1 infection. *J. Chromatogr. B* 764, 327–347.
