Chapter-6

Discussion

6. DISCUSSION

The Joint United National Program on HIV/AIDS estimated that by the end of 2005, a total of 40.3 million people worldwide were living with HIV/AIDS, the majority having been infected through heterosexual contact. It is estimated that in 2005, more than 3.1 million people died of AIDS, and 4.9 million new cases of HIV were diagnosed, including more than 700,000 children (WHO 2005). Increasingly, the public health impact of this epidemic has shifted to those regions least able to afford treatment. Fewer than 5% of those who would benefit from combination antiretroviral therapy were receiving it, even though such treatment is known to reduce the complications of infection and prolong life. Combination therapy is the current standard of care for HIV-infected persons. Duovir-N is a combination of Lamivudine, Zidovudine and Nevirapine are currently formulated in a single dose to ease the pill burden on patients receiving therapy. Method to estimate Lamivudine, Zidovudine and Nevirapine individually could be advantageous for pharmacokinetic studies and therapeutic drug monitoring in patients who are receiving either single or combination of these drugs.

In this study, we described a new RP-HPLC method with UV detection for Lamivudine, Zidivudine and Nevirapine drugs by a single

method for different classes of three drugs in bulk and tablet dosage forms. The major advantage of this method as compared to others previously published methods, because shorter chromatographic run time in contrast to other methods for such methods, Emilia Marchei et al., 2002 developed method with potassium dihydrogen phosphate (10 mM:6.5)-acetonitrile (83:17,v/v) as mobile phase with flow rate 1mL/min, for Zidovudine run time more than 5 min and Nevirapine more than 25 min, Zhou et al., 1997 carried out isocratically at a flow rate of 1mL/min with a mobile phase of phosphate buffer and methanol in the ratio of 88.3:11.7 v/v for and total run time per sample was 25 min 3TC, Kenney et al., 2000 used mobile phase methnol, acetonitrile, acetic acid, 0.1M ammonium acetate 8:1: 0.1:90.9 v/v/v/v at flow rate 1mL/min with on HPLC run time of 30 min for ZID and LAM and Alnouti et al., 2004 developed with mobile phase 5% methanol and 20mM dibasic phosphate buffer (pH 6) with run time 21 min for Lamivudine, Stefania Notari et al., developed method with KH_2PO_4 and acetonitrile as mobile phase and run time 1mL/min, the retention time for Lamivudine 4.1, for Zidovudine 16.2 and retention time for Nevirapine 16.6, so long run time of the analysis between 16 to 25 min has been reported in most of previously published method. In our method however, by eliminating of late eluting peaks and without using of any ion pair agent in the

mobile phase which its addition reduces the column life and needs to more time to stabilizing the system. In our method, used very simple solvent system as mobile phase and this reduces the run time, the retention times of Lamivudine, Zidovudine and Nevirapine were 2.7, 2.9 and 3.2 min, respectively.

Care must also be taken to analyse all standards randomly and to perform the chromatographic analysis in the shortest time possible. Acceptance criteria for calibration standards to be acceptable, each run must contain at least 75% of calibration standards back calculating to within 20% of their nominal concentrations. The calibration curve must contain at least one standard at both the lower and upper levels of quantification. The linearity and reproducibility of the various standards used for constructing calibration graphs for tablet dosage forms of Lamivudine, Zidovudine and Nevirapine were tested on six concentrations. The Lamivudine standard concentrations ranged from 5 to 100 μg/mL. The Zidovudine standard concentrations ranged from 25 to 350 μg/mL and for Nevirapine from 25 to 200 μg/mL. Slope and intercept values for each run were calculated using the equation Y= mx+C where, C is intercept, m is the slope, x the standard concentration and Y is peak area ratio, using regression analysis of the linear for

Lamivudine Y= 24.60x + 7.205 with correlation coefficient of r^2= 0.9999, for Zidovudine regression analysis of the linear Y= 22.09x + 63.47 with correlation coefficient of r^2= 0.9999 and for Nevirapine using regression analysis the of linear equation Y= 15.67x+3.213 with correlation coefficient of r^2= 0.9999.

Precision and reproducibility of the method were assessed by performing replicate analysis of standard solutions in the mobile phase and serum, Repeatability and reproducibility were characterized by RSD%. Based on these results, there was no significant difference for the assay, as tested by within-day (repeatability) and between -days (reproducibility). Repeatability was studied by injecting 3 concentrations of standard drug three times during the day. The method passed test for repeatability, as the % RSD was less than to 2%. Intermediate precision was studied by injecting 3 concentration of standard in triplicates on 3 different days the method passed the test for intermediate precision, as the % RSD was less than 1.5%. The method precision was also determined by preparing sample solution of a single batch of Lamivudine, Zidovudine and Nevirapine drug substances weighed six times and analyzing as per the proposed method at two different concentrations.

Recovery studies were also conducted with the tablets using the standard addition method. The recovery was measured by spiking the

already analyzed samples of tablets with known concentrations of standard solutions of the studied compounds. The results indicate the absence of interferences from the common pharmaceutical excipients used in the selected formulations. It is concluded that the method is sufficiently accurate and precise in order to be applied to tablet dosage forms. Accuracy was studied by injecting six times at three different concentrations of standard. The method passed the test for accuracy, as the percentage recovery for LAM 99.30 ± 06, for ZID 100.29 ±57 and NVP 99.56 ±61, in the developed method accuracy obeys according to the guidance for industry Q2B validation of analytical procedures methodology 1996.

The USP suggests that system suitability tests be performed prior to analysis. The parameters include tailing factor, capacity factor, theoretical plate number, retention time, asymmetry factor, selectivity and %RSD of peak height or area for repetitive injections. Typically, at least two of these criteria are required to demonstrate system suitability for the proposed method. Some of the tests were carried out on freshly prepared standard solutions including all three compounds. Tailing factors of 1.12, 0.962, and1.113 were obtained for LAM, ZDV and NVP respectively, the theoretical plate number (N) were 32247.057 for LAM, 31713.992 for ZDV and 110099.73 for NVP. The chromatographic conditions described ensured adequate retention

and resolution for all analytes. The retention times of LAM, ZDV and NVP were 2.7, 2.9 and 3.2 min. The results obtained from the system suitability tests satisfy the USP requirements.

For the concentration to be accepted as the Limit of Detection& Limit of Quantification LOQ & LOQ has to be less than 20% (Aymard et al., 2000). Limit of Detection & Limit of Quantification calculated according to the formula Residual Standard Deviation. LOD & LOQ were found to be 1.705 µg/mL, 5.16 µg/mL for Lamivudine, LOD 2.928 µg/mL, LOQ 8.874 µg/mL for Zidovudine and LOD 3.175 µg/mL, LOQ 9.621 µg/mL for Nevirapine.

The percentage assay of Lamivir 150 mg and Lamivir 100 mg was found to be 99.59% and 99.83%, the percentage assay of Zidovir 300 mg and Zidomax 300 mg was found to be 100.05% and 100.23%, the percentage assay of Nevimune 200 mg and Nevivir 200 mg was found to be 99.84% and 100.41% (IP, 2007).

The stability of the reference compounds and sample solutions were checked by analyzing a standard solution of the compounds in the mobile phase stored at +4°C. The results demonstrated that the working reference solutions were stable for up to a week. Stability indicating studies were performed for Lamivudine, Zidovudine and Nevirapine in different stress conditions with 0.1N NaOH, 0.1N HCl,

5% H_2O_2, Thermal and UV light at 10 µg/mL, 25µg/mL and 25 µg/mL concentration, chromatograms were completely distinguishable from the parent compounds. Lamivudine 2.710, Zidovudine 2.920 and Nevirapine 3.167 Peak areas were calculated as % RSD show less than 2%. The retention times were compared with standard bulk drugs.

The method was also applied to the determination of LAM, ZDV and NVP in dissolution rate studies. Tablets, which include 150 mg LAM, 300 mg ZDV and 200 mg NVP were investigated by the paddle dissolution method. The cumulative percentages of drug released versus the time profile were calculated for all compounds. *In-vitro* studies were performed with different pH levels gastric media, mouth pH and small intestine pH levels. The concentration of drugs release were calculated from Y= mx+C of the standard drugs of Lamivudine, Zidovudine and Nevirapine. The drug release data of the in-vitro study of tablets were analyzed with various kinetic equation like Zero order (% drug release V/s time), First order (log % remaining to release V/s time), Higuchi equation (% drug release V/s Sq.root of time) and Korsmeyer and Peppas equation (log mt/m∞ V/s lnt) (Brahmankar and Jaiswal, 1995). *In-vitro* studies the retention times of chromatograms were completely distinguishable from the parent compounds. Lamivudine 2.790, Zidovudine 2.900 and Nevirapine

3.190. The retention times were compared with standard bulk drugs. Blank plasma samples did not give any peak at the retention times of LAM, ZID and NVP.

Validation with spiked rabbit serum samples was carried out by use of the appropriate calibration equations obtained with spiked serum samples. Analysis of drugs from serum samples usually requires extensive time-consuming sample preparation and the use of organic solvents. In this study, the serum proteins were precipitated by the addition of 10% trichloroacetic acid followed by centrifugation. The supernatant was diluted with mobile-phase and samples, including various concentrations of LAM, ZDV and NVP directly injected into the LC column. The concomitant therapeutic agents most likely to be encountered in the plasma of animal studies were screened under the HPLC assay conditions. Pharmacokinetic *in vivo* studies were performed for Lamivudine, Zidovudine and Nevirapine. In order to under the mechanism and kinetics of drug release by rabbits animal studies in different groups, the drug release data of the in-vivo studies were analyzed with various kinetic equation like Zero order (% drug release V/s time), First order (log % remaining to release V/s time), Higuchi equation (% drug release V/s Sq.root of time) and Korsmeyer and Peppas equation (log $mt/m\infty$ V/s lnt). The concentration of drug release were calculated from Y= mx+C of the

standard drugs of LAM, ZID and NVP and *in-vivo* parameters also calculated like Maximum Time (Tmax), Maximum Concentration (Cmax), Volume of distribution (Vd), Area Under Curve (AUC), Clearance (CL) and Half life ($t_{½}$) (Brahmankar and Jaiswal, 1995). *In-vivo* studies the retention times of the biological products were completely distinguishable from the parent compound. The retention times of Lamivudine 2.623, Zidovudine 3.017 and Nevirapine 3.320 respectively *in-vivo* studies. The retention times were compared with standard bulk drugs.

The proposed single method for different classification of NRT and NNRTs of Lamivudine, Zidovudine and Nevirapine in bulk and tablet dosage forms was simple, precise, accurate, rapid and economical. The method is linear in the concentration range reported. The developed method is free from interference due to the excipients present in various brands of tablets and can be used for routine either individual or simultaneous quantitative estimation of Lamivudine, Zidovudine and Nevirapine in tablets.

The developed method applied to *in-vitro* & *in-vivo* studies which is potentially suitable for drug monitoring and determination of pharmacokinetic profiles.