# Simple and rapid method development and validation of RP-HPLC method for the determination of zidovudine in human plasma

**Nalanda R. Baby [a], Srinivasa A. Rao [a],*, Pavani S. Naidu [a], Druga Surla [a], Nagamani Bolla[b]**

[a] *Department of Pharmaceutical Analysis and Quality Assurance, Shri Vishnu College of Pharmacy, Vishnupur, Bhimavaram 534202, West Godavari-D.T., Andhra Pradesh, India*
[b] *Pharmaceutical Research & Development Laboratory, Corpuscle Research Solutions, Visakhapatnam 530017, Andhra Pradesh, India*

### ABSTRACT

*Background*: Zidovudine, as monotherapy or in combination with other antiretroviral agents, remains a first-choice therapy for the prophylaxis of mother-to-child HIV transmission. Since sufficient bioanalytical methods have not been reported officially for the quantitative estimation of zidovudine, there is necessity for investigation of new analytical method for quantitative estimation of zidovudine in human plasma. *Objective*: An isocratic, simple, precise and accurate HPLC-UV method was developed and validated for determination of zidovudine in human plasma using nevirapine as an internal standard to support anti-retroviral pharmacology programs. *Materials and methods*: The extraction process involved a liquid-liquid extraction and were chromtographed on a Phenomenex $C_{18}$ (250×4.6 mm i.d., 5 µm particle size) column at ambient temperature using a mobile phase consisting of methanol and 0.1% orthophosphoric acid (40:60 % v/v), the flow rate of 1.0 mL/min and UV detection at 265 nm. The method was found to be specific for AZT and no endogenous biological substances were found to be interfering with peaks of drug and internal standard. Results: The method showed good linearity in the range of of 81.70-5263.55 ng/mL and correlation of coefficient ($r^2$) was found to be 0.99784. The day co-efficient of variations was 0.37-4.95%. The overall recovery for AZT was found to be 55.48 % respectively. Stability data revealed that the drugs were stable in plasma under various test conditions. *Conclusion*: The proposed method was applicable for clinical therapeutic drug monitoring programs of AZT and useful in the determination of pharmacokinetic profile and bioequivalence studies of HIV research.

**KEYWORDS:** HPLC-UV, Human plasma, Zidovudine, Nevirapine, Liquid-liquid extraction.

## 1. INTRODUCTION

Zidovudine (AZT), is 1-(3-azido-2, 3-dideoxy-ß-D-erythro-pentofuranosyl)-5-methylpyrimidine-2,4-(1H,3H)-dione (Fig. 1), used in antiretroviral medication to prevent and treat HIV/AIDS. Zidovudine was one of the first 2',3'-dideoxynucleoside, belongs to reverse transcriptase inhibitors (NRTIs) class, inhibiting the human immunodeficiency virus (HIV) replication in vitro, by inhibiting the enzyme, viral reverse transcriptase. Zidovudine also used to treat neurological diseases associated with HIV disease with some success, because it readily crosses the blood-brain barrier. AZT has been also used in association with improvements in neuro-developmental and growth velocity in HIV-infected children. The use of zidovudine as a prophy-

laxis has also been suggested, but the value of this is questionable. The combination of zidovudine with other agents, such as acyclovir and interferon, called highly active antiretroviral therapy (HAART) exhibited synergistic effect on the anti-HIV activity, with reduced drug toxicity. Zidovudine was the first drug to be approved for treatment of HIV infection[1]. FDA approval of zidovudine for prevention of HIV transmission from mothers to infants was granted in 1994[2].
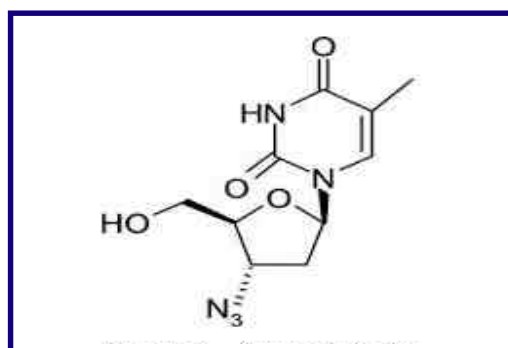
**\*Corresponding author.**
*Dr.Srinivasa A. Rao*
*Professor & HOD*
*Dept. of Pharmaceutical Analysis & QA*
*Shri Vishnu College of Pharmacy*
*Vishnupur, Bhimavaram - 534202*
*West Godavari Dist., A.P., India.*

**Figure 1. Chemical structure of zidovudine**

The effectiveness of AZT in the treatment of HIV infection is due to its selective affinity for HIV reverse transcriptase as opposed to human DNA polymerases. AZT use was a major breakthrough in AIDS therapy in the 1990s that significantly altered the course of the illness and helped to destroy the notion that HIV/AIDS was a death sentence[3]. Zidovudine is still widely used for antiretroviral therapy in combination with other antiretroviral agents[4]. AZT is rapidly eliminated from the body by metabolic conjugation to the glucuronide (about 75% of the dose) and by excretion of the unchanged drug in the urine (about 20% of the dose). Only the unchanged drug is thought to be the precursor of the active species, zidovudine triphosphate. The hematological toxicity of AZT, which has been found to be both dose- dependent and reversible, is of serious concern. For this reason it is likely that plasma concentration of AZT may, in the future, be monitored in order to individualize drug dosage regimen[5]. Nanotechnology applied to the development of new drug delivery systems, has emerged as an effective means of targeting therapeutic agents to various target sites and has created new alternatives for AIDS treatment. Various analytical methodologies have been developed for the evaluation of this delivery system, both *in vitro* and *in vivo*. Several studies have tested the safety of new antiretroviral drug delivery systems in animal models also. Due to the corresponding increase in preclinical studies, it is essential to develop and validate analytical methods to quantify the antiretroviral drugs in biological fluids[6].

Literature survey revealed that various methods have been reported for determination of AZT, in particular using chromatography[7-13]. High performance liquid chromatography (HPLC) and Radioimmunoassay (RIA) was developed to determine intracellular ZDV and anabolite concentrations of 10 PBMC samples taken from HIV positive patients on ZDV treatment[14]. New technique was developed on fluorescence-based detection i.e radiometric analysis to visualize zidovudine-incorporated DNA by click chemistry[15]. Other techniques such as micellar liquid chromatography[16] and absorptive stripping voltammetry analysis[17], fluorescence derivatization method were used to quantify the AZT[18]. Determination of Zidovudine with other Anti-retroviral agents in biological fluids done by various extraction process have been published, including solid-liquid extraction[19], liquid-liquid extraction[20], column switching[21], deproteination[22], FT-IR and UV[23]. A new method has been reported using surface-enhanced Raman scattering (SERS) and density functional theory (DFT) based on the molecular electrostatic potential (MEP) and the selective Raman bands enhancement[24]. Simultaneous estimation of AZT in biological fluids and drug products using high performance thin layer chromatography (HPTLC)[25], LC- ESI-MS/MS spectrometric method have been reported[26]. Also, anion exchange solid phase extraction and liquid chromatography–tandem mass spectroscopy[27], and LC–APCI-MS–MS methods have been reported for the detection of AZT and its active forms in aquatic environments and in plasma[28].

There is no article concerning individual, simple and cost effective validated method in human plasma for AZT. So, the aim of present study was to establish a validated method based on HPLC-UV that is capable of analyzing AZT in both solution and human plasma in order to facilitate routine pharmacokinetic and bioequivalence as well as nanotechnology studies of AZT.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

Pharmaceutical grade Zidovudine (Heterodrugs Ltd., Hyderabad, India, 99.5%) and Nevirapine as internal standard (Aurobindo Pharma Ltd., Hyderabad, India, 99.6%) were used as working standards after confirming their purity. Methanol and Methyl-t-butyl ether (HPLC grade) were purchased from Merck Ltd., Mumbai, India. All the other reagents used were analytical grade. The lyophilized human plasma was purchased from Sri Laxmi Sai Clinicals, Hyderabad, India. Milli-Q water was used throughout the study. Methanol: water (50:50 % v/v) used as diluent in experimentation.

### 2.2. Equipment

The HPLC (Shimadzu, Japan) instrument was equipped with a model binary LC 10ATVP pumps, SIL 10ADVP Autosampler, CTO 10 AVP column oven, a phenomenex $C_{18}$ column (150 mm × 4.6 mm i.d, 5µm) and an SPD 10AVP UV- VIS detector. All the components of the chromatographic system controlled using SCL-10AVP system controller. Data acquisition was performed by using LC solutions ver. 1.23 SP 1 software.

### 2.3. Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a Phenomenex $C_{18}$ column. The mobile phase was prepared by a mixture of methanol: orthophosphoric acid (40:60 % v/v) with a flow rate of 1.0 mL/min was employed. The detector wavelength was set at 265 nm. The injection volume was 20 µL and the total run time was 10 min.

### 2.4. Preparation of standard & Quality control samples

Stock standard solutions of drug (1.15 mg/mL and internal standard (IS) (1 mg/mL) were separately prepared in diluent and stored at below 10 °C. Working solutions were prepared by appropriate dilution in diluent just before use. All solutions were stored in darkness at 4°C. Volumes of 25 µL of AZT working solutions and 25 µL of IS (50

µg/mL) were added to 475 µL of drug-free human plasma to obtain drug concentration levels of 81.70, 163.40, 480.59, 1029.83, 2059.65, 3089.48, 4119.30, and 5263.5 ng/mL. Similarly, quality control (QC) samples were also prepared separately and pooled at three different concentration levels (245.10, 2631.78, 3718.81 ng/mL) as low, medium, high, respectively. The samples were stored in a freezer at -20 °C until analysis.

### 2.4.2. Treatment of Plasma Samples

The stored plasma samples were thawed at room temperature before processing. The plasma samples were centrifuged at 5000 rpm for 10 min. An aliquot (1.0 mL) was pipette into a 2.0 mL eppendorf micro centrifuge tube and methyl-t-butyl ether (1 mL) was added and vortex mixed for 2 min. The mixture was again centrifuged at 5000 rpm for 5 min. The supernatant layer was transferred into another tube, evaporated to dryness under nitrogen atmosphere at 40 °C and the dried residue was reconstituted with 300 µL of mobile phase, vortexed thoroughly. Volume of 20 µL was injected into HPLC system.

### 2.5. Validation of the bioanalytical method

The method was validated by the determination of the following parameters: specificity, linearity, recovery, accuracy, precision, lower limit of quantification (LLOQ) and stability studies according to the currently accepted US food drug administration (FDA) bioanalytical method validation guidance[29].

### 1.5.1. Specificity and selectivity

Selectivity is the ability of an analytical method to differentiate the analyte in presence of other components in the sample. Before the preparation of the pooled calibration standards and QC samples, six lots of blank plasma were screened for matrix effects or interferences. The interference from individual blank plasma in the LC-UV chromatograms at the retention times of the studied drug and IS with or without drug and IS was investigated to ensure the specificity of the method.

### 2.5.2. Calibration curve

The calibration curves were constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS), and eight concentrations of the studied drugs including the LLOQ, ranging from 81.70-5263.5 ng/mL of peak area ratios (PAR) against the standard concentration and the linearity evaluated by least squares regression analysis. PAR gives the following equation: $PAR = Slope \times C + Intercept$. The slope and the intercept are determined from the determined PAR and the nominal concentration of the drug. The unknown AZT concentrations are determined from this equation.

### 2.5.3. Precision and accuracy

The precision of the method based on intraday variability was determined by replicate analysis of the calibration standards in the same day. The reproducibility was taken as the inter-day variability and was determined by replicate analysis of the calibration standards in different days with one replicate being analyzed each day. The percentage coefficient of variation values (%CV) were calculated from the ratios of the standard deviation (SD) to the mean. The accuracy of the analytical method describes the closeness of the mean test results obtained by the method to the true value of the analyte. The evaluation of precision was based on the criteria that the deviation of each concentration level should be within ± 15%, except for the LLOQ, for which it should be within ± 20%. Similarly for accuracy, the mean value should not deviate by ± 15% of the nominal concentration, except the LLOQ, where it should not deviate by ±20 % of the nominal concentration.

### 2.5.4. Lower limit of quantification (LLOQ)

Lowest standard concentration on the calibration curve should be accepted as the limit of quantification if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compares to blank response and analyte peak (response) should be identifiable, discrete and reproducible with precision of 20 % and accuracy of 80-120%.

### 2.5.5. Recovery

Recovery from plasma was determined for QC samples (LQC, MQC and HQC) of drug by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration. The percentage of the drug recovered from the plasma samples was determined by comparing the peak area ratio after extraction, with those of non-extracted sample containing same concentrations of the drugs as in plasma.

### 2.5.6. Stability

The concentration of the studied drugs after each storage period was related to the initial concentration as zero cycle (sample that were prepared and processed immediately). The samples were considered as stable if the standard deviation (expressed as percentage bias) from the zero cycles was within ± 15%.

### 2.5.6.1. Freeze- thaw stability

The freeze-thaw stability of the studied drugs was determined at low and high QC samples (n=6), over three freeze-thaw cycles stored at − 80 °C within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and the results were compared with that of zero cycle.

### 2.5.6.2. Bench top stability

The stability of the low and high unprocessed QC samples were maintained at a temperature of 25° C for 9 h and the samples were analyzed and the results were compared with that of zero cycle.

### 2.5.6.3. Long term- stability

The stability of the low and high QC samples was frozen at $-20\,°C$ for 15 days. The samples were analyzed and results were compared with that of zero cycle.

### 2.5.6.4. In-injector stability

The stability of the low and high QC samples was stored at 4 °C for 48 h in the auto-sampler. The samples were analyzed and results were compared with that of zero cycle.

### 3. RESULTS AND DISCUSSION

The developed HPLC method was optimized for the analysis of zidovudine in human plasma. To obtain the best chromatographic condition, different columns like $C_8$, $C_{18}$ of Agilent, Zorbax, Polaris and Phenomenex were used. The run time of analysis was higher when a longer reverse phase column ($250 \times 4.6$ mm i.d,) was used. The resolution between the peaks was decreased and peaks were not of acceptable shape when the experiment was performed using a shorter column ($50 \times 4.6$ mm i.d,). However better resolution, less tailing and high theoretical plates were obtained with Phenomenex column $C_{18}$ ($150 \times 4.6$ mm, 5 µm) column. and mobile phases composed of buffer system like orthophosphoric acid, phosphate and acetate buffer with different pH ranged from 3-7 and organic modifier like methanol and acetonitrile were tested to provide sufficient selectivity and sensitivity in short separation time.. There was no interference in the drug and the internal standard, from the extracted blank plasma. The peak shape and symmetry were found to be good when the mobile phase composition of methanol: orthophosphoric acid (40:60 v/v) was used with better resolution of the drug and internal standard. The influence of both organic modifier (methanol) concentration and aqueous portion was carefully studied. Increasing organic modifier concentration not only improves peak shape and decreasing the run time but also decreasing method specificity due to the interference of the zidovudine peak with endogenous biological substances. A mobile phase containing aqueous portion greater than 85% led to very late elution and poor peak shape for AZT with broad and unacceptable asymmetry factor. Extraction methods were initially attempted using protein precipitation technique. Organic solvents such as acetonitrile and/or methanol were used as reagents for protein precipitation[30]. Initial experiments of protein precipitation were done using 1:3 ratio of plasma: organic solvents. The recovery of the AZT was poor while that of the internal standard was relatively unchanged as com-

pared with liquid-liquid extraction. Various solvents such as ethyl acetate, diethyl ether, 100% methyl-t-butyl ether were used for extraction. The highest recovery from the plasma samples was obtained with t-butyl methyl ether. The best chromatographic separation condition occurred on Phenomenex $C_{18}$ column with a mobile phase consisting of 0.1% orthophosphoric acid (pH 3.5) - methanol (60:40, v/v) at a flow rate of 1 mL/min and UV detection at 265 nm. The retention time for extracted peaks of AZT and IS were 4.18 min and 8.25 min respectively.

### 3.1. Selectivity

The selectivity of the present method was evaluated by analyzing the blank plasma obtained from different blood donors. Six different lots of blank plasma were chromatographed to check for endogenous components which interfere with zidovudine and internal standard (nevirapine). Each sample was eluted three times individually and they found to be free of co-eluting peaks at the retention time range of drug and internal standard. There was also a good resolution between the peaks of zidovudine and internal standard (Fig. 2 and 3).
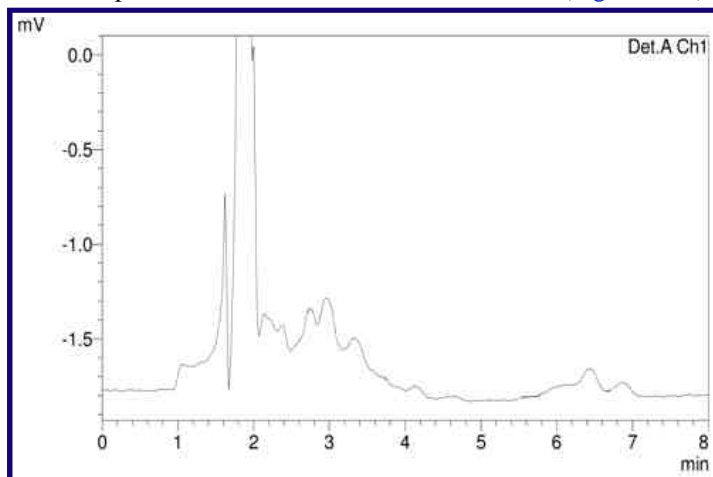


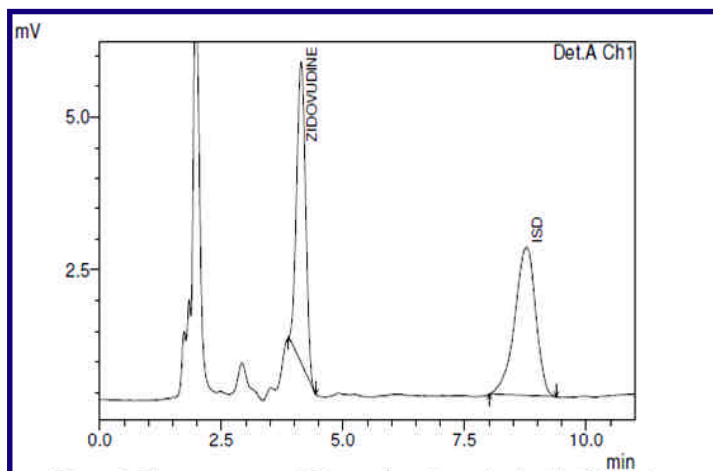**Figure 2. Chromatogram of Blank plasma**



**Figure 3. Chromatogram of Plasma spiked samples with Zidovudine and internal standard (Nevirapine)**

## 3.2. Linearity

The linearity of the method was determined by a weighted ($1/X^2$ where X is concentration) least square regression analysis of the standard plots associated with the eight point standard curve for zidovudine. The calibration curve was obtained by plotting chromatographic peak area ratio (zidovudine/nevirapine) versus concentration of zidovudine. The calibration line was linear in the range of 81.70 to 5263.55 ng/mL of the drug (Table 1, Fig. 4). A straight-line fit made through the data points showed a constant proportionality with minimal data scattering. The regression coefficient ($r^2$) ranged from 0.99723 to 0.99907.

**Table 1. Results of Regression analysis of the linearity data**

| Parameters | Zidovudine |
|---|---|
| Linearity range (ng/mL) | 81.70-5263.55 |
| Quantification limit (ng/mL) | 81.7 |
| Number of experiments (n) | 6 |
| Slope | 0.00017 |
| Intercept | $0.015825 \pm 0.003$ |
| Correlation coefficient ($r^2$) | $0.99784 \pm 0.001$ |



**Linear calibration curve for Zidovudine using HPLC-UV using Nevirapine as an Internal Standard Method**

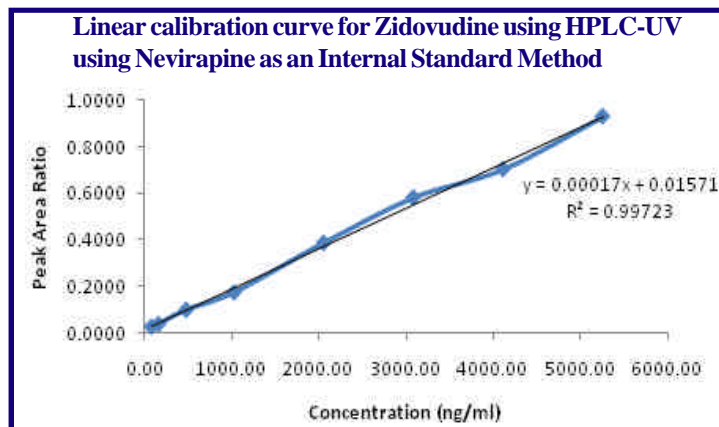$y = 0.00017x + 0.01571$
$R^2 = 0.99723$

**Figure 4. Calibration curve of Zidovudine**

## 3.3. Carryover test

A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octadecyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

## 3.4. Precision and Accuracy

The precision and accuracy of the method were assessed by analyzing six replicate QC samples at low, medium and high concentration. The intra-day and inter-day precisions were measured as the coefficient of variation (CV) expressed as percentage over the concentration. This is presented in (Table 2) for the intra-day and inter-day precisions. The results indicated an acceptable precision for all concentration assayed for both intra-day and inter-day samples. The %

CV of AZT ranged 0.37 % and 1.4 to 4.1 % for both intra-day and inter-day precisions respectively. The low values of % CV reflect the precision of the assay method.

Accuracy of the method was determined by replicate analysis of six sets of QC samples and comparing the difference between the spiked value (nominal value) and that actually found. The accuracy was expressed as the % of nominal concentration. The % nominal concentrations of AZT were ranged from 100.3 to 105.9 % and 100 to 106.34 % for intra-day and inter-day respectively. These high values of the % nominal concentration reflect the accuracy of the assay method.

**Table 2. Intra-day and inter-day accuracy and precision of HPLC assay**

| | Concentration added (ng/mL) | | |
|---|---|---|---|
| | 245.1 | 2631.78 | 3718.81 |
| **Intra-day** | | | |
| Mean | 249.42 | 2786.92 | 3730.4 |
| SD | 0.92 | 10.4 | 13.88 |
| % CV | 0.37 | 0.37 | 0.37 |
| % RE | 1.7 | 5.8 | 0.3 |
| Accuracy % | 101.76 | 105.9 | 100.31 |
| **Inter-day** | | | |
| Mean | 248.65 | 2778.32 | 3719.08 |
| SD | 5.54 | 65.2 | 154.8 |
| % CV | 5.54 | 2.35 | 4.16 |
| % RE | 1.4 | 5.5 | 0.007 |
| Accuracy % | 101.45 | 105.57 | 100.01 |

*Each mean value is the result of triplicate analysis*

## 3.5. Recovery

The absolute recovery of the studied drug was determined by comparing the peak area ratio of the QC sample spiked in human plasma and defined in three runs with those of extraction plasma sample with known amount of analytes. The mean recovery of AZT from plasma spiked samples, in terms of LQC, MQC and HQC levels were respectively, 60.7%, 56.2% and 49.5%. The overall recovery of AZT was 55.48%. Table 3, indicated the high ability of the proposed method to recover the studied drug from human plasma.

**Table 3. Recovery studies of Zidovudine in Human plasma at three concentrations (n = 6 )**

| Concentration (ng/mL) | % Recovery (mean ± SD) | %CV |
|---|---|---|
| 245.1 | $60.7 \pm 4.48$ | 7.38 |
| 2631.78 | $56.2 \pm 1.25$ | 2.22 |
| 3718.81 | $49.5 \pm 0.47$ | 0.95 |

## 3.6. Stability

Stability of the sample was determined in various phases of method.

The stability includes freeze thawed stability, Bench top stability, long-term stability, in-injector stability.

The stability of the studied drugs in human plasma was assessed by analyzing six replicate QC samples at the low, and high concentration levels at room temperature over 9 h (Bench top stability). The measured concentrations of the drugs in these QC samples kept at room temperature for 9 h were compared with the corresponding QC sample freshly prepared and proceed immediately. The results in Table 4 indicated that the studied drugs were stable for at least 9 h in human plasma when stored at ambient temperature. On the other hand, QC samples experiencing three freeze-thaw cycles (freeze-thaw stability) were analyzed together. The results indicated that the stability of the studied drug in human plasma over three freeze-thaw cycles. Also the studied drug showed the long term-stability in human plasma when stored at −20 °C for 15 days (where the whole samples were frozen, thawed and completely analyzed) when compared with the freshly prepared QC samples. The studied drug were also stable, when stored at 4 °C for 48 h in the auto sample and compared with the freshly prepared sample as in-injector stability. All the above stability studies indicated that the samples in various phases were within the acceptance limits.

**Table 4. Summary of stability of Zidovudine in Human plasma at varying condition**

| Add concentration (ng/mL) | Theoretical concentration Zidovudine | |
|---|---|---|
| | 245.1(LQC) | 3718.81(HQC) |
| (a) Three freeze-thaw cycles | | |
| Mean | 246.41 | 3720.74 |
| SD | 3.9 | 125.3 |
| CV % | 1.58 | 3.37 |
| RE % | 0.5 | 0.05 |
| ( b) Bench top stability (9h) | | |
| Mean | 248.09 | 3752.84 |
| SD | 7.03 | 116 |
| CV % | 2.8 | 3.09 |
| RE % | 1.21 | 0.9 |
| (c)Long term stability after 15 day at-20 °C | | |
| Mean | 251.31 | 3819.66 |
| SD | 12.03 | 140.5 |
| CV % | 4.78 | 3.68 |
| RE % | 2.5 | 2.5 |
| (d) In-injector stability (48h) at 4 °C | | |
| Mean | 249.9 | 3785.9 |
| SD | 12.45 | 121.62 |
| CV % | 4.98 | 3.21 |
| RE % | 1.9 | 1.8 |

*Each mean value is the result of triplicate analysis*

## 4. CONCLUSION

The optimized HPLC-UV method is selective, accurate, precise and reproducible. The method is linear over a wide range and utilizes a mobile phase which can be easily prepared. The simplicity of the technique was used small volume of plasma sample preparation for rapid liquid-liquid extraction process without any contamination, making it widely applicable for the HIV/AIDS clinical therapeutic drug monitoring programs. The result of this study indicates that the method would be applicable to toxicokinetic, pharmacokinetic, bioavailability and bioequivalence studies. It can be concluded that the method is suitable for the routine quantification of zidovudine in biological fluids.

## Conflict of interest

We wish to confirm that there is no known conflict of interest associated with this research work.

## REFERENCES

1. Kamali F. Clinical pharmacology of zidovudine and other 2´,3´-dideoxynucleoside analogues. Clin Investig. 1993; 71(5):392-405.
2. Connor EM, Sperling RS, Gelber R, Kiselve P, Scott G, O´Sullivan MJ, Vandyke R, Bet M, Shearer W, Jacobson RL. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N Engl J Med. 1994; 331(18):1173-1180.
3. Richard JW. Drug discovery for human immunodeficiency virus infection: conquests and hurdles. J. Antimicrob Chemother. 1996; 37:151-159.
4. De Clercq E. Antiviral drugs in current clinical use. J. Clin.Virol. 2004; 30:115-133.
5. Edward PA, Keith H, Linda M, Alejo E, Henry HB, Courtney VF. Pharmacokinetics and safety of concentration-controlled oral zidovudine therapy. Pharmacotherapy. 1997;17 (3):424-430.
6. Ankita AN, William JT, Alekha KD. Current analytical methods used in the in-vitro evaluation of nano-drug delivery systems. Curr. Pharm. Anal. 2012; 8(4):358-366.
7. Underberg WJM, Underberg-chitoe, Bekers O, Meenhorst PL, Beijnen JH. A rapid, simple and accurate method for the bioanalysis of zidovudine. Int. J. Pharm. 1989; 50:175-179.
8. Bin F, Stewart JT. Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC. J. Pharm. Biomed. Anal. 2002; 28(5):903-908.
9. Yazen A, Catherine AW, Micheal GB. Simultaneous determi-

nation of zidovudine and lamivudine from rat plasma, aminotic fluid and tissues by HPLC. Biomed. Chromatogr. 2004; 18(9):641-647.

10. Vibhuti K, Vivek A, Chandrabose K, Piyush T. Simultaneous quantitative determination of zidovudine and nevirapine in human plasma using isocratic, reverse phase high performance liquid chromatography. Trop. J. Pharm res. 2009; 8(1):79-86.

11. Megha S, Pragya N, Surendra J, Deepti J. Simple and rapid RP-HPLC method for simultaneous determination of acyclovir and zidovudine in human plasma. J. AOAC. Int. 2010; 93(5):1460-1467.

12. Lakshmi KS, Kusuma KS, Visala G, Kumar VB, Mallikarjun B, Nagaraju G, Rambabu K. Method development and validation of RP-HPLC method for determination for zidovudine. Int. J. Res. Pharm. Chem. 2011;1(3):677-680.

13. Nandi U, Das A, Roy B, Choudhury H, Gorain B, Pal TK. Development and validation of an HPLC-UV method for simultaneous determination zidovudine, lamivudine, and nevirapine in human plasma and its application to pharmacokinetic study in human volunteers. Drug Test. Anal. 2013; 5(6):485-491.

14. Peter K, Lalezari JP, Gambertoglio JG. Quantification of zidovudine and individual phosphates in peripheral blood mononuclear cells by a combined isocratic high performance liquid chromatography radioimmunoassay method. J. Pharm. Biomed. Anal. 1996; 14(4): 491-499.

15. Minseob K, Jongmin P, Heeseon A, Seung HM. Ratiometric analysis of zidovudine (ZDV) incorporation of reverse transcriptases or polymerases via bio-orthogonal click chemistry. Chem. Commun. 2011; 47(27):7614-7616.

16. Casas-Breva J, Peris-Vicente M, Rambla A, Carda BS, Esteve RJ. Monitoring of HARRT regime anti-retroviral in serum of Acquired immunodeficiency syndrome patients by micellar liquid chromatography. Analyst. 2012; 137(18):4327-4334.

17. Arnaldo AC, Ricardo QA, Nicolas AR, Eliane MM, Percio AMF. Determination of Anti-retroviral drug zidovudine in Diluted Alkaline Electrolyte by Adsorptive Stripping Voltammetry at the Mercury Film Electrode. Am. J. Anal. Chem. 2011; 2(2): 223-234.

18. Maede Y, Kishikawa N, Ohyama K, Wada M, Ikeda R, Kuroda N. Fluorescence derivatization method for sensitive chromatographic determination of zidovudine based on the Huisgen reaction. J. Chromatograph. A. 2014; 1355:206-210.

19. Tarinas A, Tapanes RD, Ferrer G, Perez J. Validation of high-performance liquid chromatography methods for determination of zidovudine, stavudine, lamivudine and indinavir in human plasma. Farm. Hosp. 2007; 34(4):243-247.

20. Geetha R, Hemanthkumar AK, Kumaraswami V, Soumya S. A simple and rapid liquid chromatography method for simultaneous determination of Zidovudine and nevirapine in plasma. J. Chromatograph. B. 2006; 843(2):339-344.

21. Lacroix C, Hoang TP, Wojciechowski F, Duwoos H, Nouveau J, Diquet B. Simultaneous quantification of zidovudine and its metabolites in serum and urine by high-performance liquid chromatography using a column switching technique. J. Chromagraph. 1990; 525(1):240-245.

22. Rubiana MM, Maria PDG. Reverse phase HPLC determination of zidovudine in rat plasma and its pharmacokinetics after a single intranasal dose administration. Bio. Res. 2009; 42(3):357-364.

23. Ashok P, Sagar DV, Chandrakant GB. A quantitative analysis of zidovudine containing formulation by FT-IR and UV spectroscopy. Anal. Methods. 2010; 2(11):1756-1763.

24. Nicoleta EM, Andrada V, Krisztian H, Vasile C, Nicolae L. Surfaced-enhanced Raman and DFT study on zidovudine. Spectrosc. 2011; 26(4-5):311-315.

25. Palani V, Mahesh D. Development and validation of normal-phase HPTLC method for simultaneous analysis of Lamivudine and Zidovudine in fixed-dosage combination tablets. J. Pharm. Anal. 2012; 2(2):152-155.

26. Estrela RC, Salvadori MC, Suarez KG. A rapid and sensitive method for simultaneous determination for lamivudine and zidovudine in human serum by on-line solid-phase extraction coupled to liquid chromatography/tandem mass spectroscopy detection. Rapid Commun. Mass Spectrum. 2004; 18(10):1147-1155.

27. Tracy K, Lane B, Peter LA, Thomas D, Michelle R, Courtney VF. Quantification of zidovudine triphosphate concentration from human peripheral blood mononuclear cells by anion exchange solid phase extraction and liquid chromatography-tandem mass spectroscopy; an indirect quantitation methodology. J. Chromatogr. B. 2006; 831:248-257.

28. Peng Y, Zhenkun G, Xin G, Liling R, Hang C, Xingyan Z. LC-APCI-MS-MS Analysis of Zidovudine in Human plasma, and Application to a Bioequivalence Study. Chromatographia. 2010; 72(1):151-155.

29. *Guidance for Industry, Bioanalytical Method Validation.* U.S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research; 2001.

30. Polson C, Sarkar P, Incledon B, Raguvaram V, Grant R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2003; 785:263.