Journal of Pharmaceutical Research Vol. 9, No. 2, April 2010 : 56-59.

A Rapid and Sensitive RP-UPLC Method for Simultaneous Determination of Zidovudine, Lamivudine and Nevirapine in Tablet Dosage Form

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Received on : 31.08.2009 Revised : 01.02.10 Accepted : 22.02.10

ABSTRACT

The objective of the current study was to develop a validated ultra performance liquid chromatographic (UPLC) method for the quantitation of the novel antiretroviral agents zidovudine, lamivudine and nevirapine in tablet dosage form. The method utilizes sample preparation step followed by separation on a waters C_{18} column consisting high strength silica (ACQUITY UPLC HSS-T3), 100 × 2.1 mm, 1.8 mm particle size, in gradient mode using 0.1%, v/v trifluoroacetic acid in water and acetonitrile as the mobile phase. The analytical column was thermostated at 50°C and flow rate was set at 0.4 mL/min. The eluted peaks were detected by photo diode array (PDA) detector at a wavelength of 266 nm. The method was validated with respect to linearity, accuracy, precision and robustness. The responses were linear over the ranges of 100-500, 50-250 and 100-180 mg/mL for zidovudine, lamivudine and nevirapine, respectively. The limit of detection and limit of quantification were found 0.279, 0.068, 0.360 and 0.846, 0.205, 1.091 mg/mL for zidovudine, lamivudine and nevirapine, respectively. The utility of the procedure was verified by its application to formulations and found that the developed method is fast, accurate, precise, selective and reproducible.

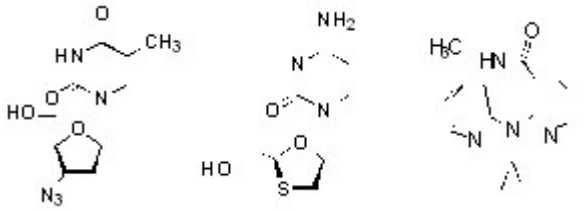
Keywords: *Zidovudine, Lamivudine and Nevirapine; UPLC; Method development and validation.*

INTRODUCTION

Zidovudine (AZT), 3[2]-Azido-3[2]-deoxythymidine, is the first antiretroviral drug used for the treatment of HIV infection. It is anabolized intracellularly to its 5[2]-triphosphate which acts as a potent inhibitor of HIV reverse transcriptase and a terminator of growing proviral DNA[1].

Lamivudine (3TC), 4-amino-1-((2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl)pyrimidin-2(1H)-one is a pyrimidine analog reverse transcriptase inhibitor; active against HIV-1, HIV-2 and hepatitits B virus[2]. The (-) enantiomer has less cytotoxicity, greater antiviral activity than the (+) enantiomer and rapidly absorbed with a bioavailability of approximately 80 %[3].

Nevirapine (NVP), 11-Cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido [3,2-*b*:2□,3□-*e*][1,4] diazepin-6-one, is a potent and selective non competitive inhibitor of the reverse transcriptase (Figure 1).



Zidovudine (AZT) Lamivudine (3TC) Nevirapine (NVP)

Fig. 1. *Structural formulae of zidovudine, lamivudine and nevirapine.*

Literature survey reveals several methods used for the quantitative determination of AZT, 3TC and NVP individually or in combination by HPLC[4-6], HPTLC[7] etc. Besides, simultaneous quantification of several antiretroviral agents including these three drugs has been reported by a solid-liquid extraction procedure using RP-HPLC system[8].

The aim of this work was to develop a UPLC method for the simultaneous quantification of AZT, 3TC and NVP in antiretroviral FDCs without the necessity of sample pre-treatment. This paper describes the development and validation of reversed phase UPLC method, using PDA detector, for the simultaneous determination of AZT, 3TC and NVP in FDC tablets. The method appears to be suitable for quality control in pharmaceutical industry due to its sensitivity, selectivity and lack of excipients interference.

EXPERIMENTAL

Apparatus

Chromatography was performed with a Waters, Acquity UPLC (Waters, Milford, MA, USA) chromatographic system equipped with a binary solvent manager, sample manager (auto-sampler), inline degasser, and 2996 photo diode array (PDA) detector. Empower-2 software was used for LC peak integration along with data acquisition and data processing. The mobile phase was degassed by sonication with an ultrasonics bath

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(DTC 503; Vetra, Italy). The standard substances were weighed on a Sartorious analytical balance (ATS- 220).

Reagents and Materials

Pure AZT, 3TC and NVP were provided by Aurbindo Pharma Ltd. (Hyderabad, India) with 98.5, 99.1 and 99.6 % purity, respectively. Combination product ZIDOVEX LN (label claim: 300 mg AZT, 150 mg 3TC and 200 mg NVP per tablet) were also provided by Aurbindo Pharma Ltd. Hyderabad, India.

Acetonitrile and methanol of HPLC grade were purchased from E. Merck Ltd. (Mumbai, India). Trifluoroacetic acid and other chemicals used were of analytical grade. The water used was of Milli-Q grade purified by a Milli-Q UV purification system (Millipore, Bedford, MA, USA).

Preparation of standard solutions

About 50 mg of AZT, 25 mg of 3TC and 32.5 mg of NVP working standards were accurately weighed into a 100 ml volumetric flask, dissolved in 10 mL of methanol and diluted to volume with water. Further, 10 mL of this solution was diluted to 25 mL with water to obtain a concentration of 200 mg/mL of AZT, 100 mg/mL of 3TC and 130 mg/mL of NVP, respectively.

Preparation of sample solution

Twenty tablets were accurately weighed, their average weight was determined, and the tablets were powdered in a glass mortar. A quantity of powder equivalent to 50 mg of AZT was dissolved in 10 mL of methanol and the mixture was sonicated for 15 min. The resulting mixture was filtered by using a 0.45 mm nylon filter paper in a 100 mL volumetric flask, and the filtrate was diluted to volume with water to obtain a solution containing AZT (500 mg/mL), 3TC (250 mg/mL) and NVP (325 mg/mL). Further, 10 mL of this solution was diluted to 25 mL with water to obtain a working solution containing AZT (200 mg/mL), 3TC (100 mg/mL) and NVP (130 mg/mL) for analysis.

Chromatographic conditions

Chromatographic separations were performed with gradient elution. All the determinations were performed at 50°C using Waters C_{18} column consisting high strength silica (ACQUITY UPLC HSS-T3), (100 × 2.1) mm x 1.8 mm particle size with the injection volume of 2 ml. Two mobile phase components were as follows: *mobile phase* (A) comprising of 0.1 %, v/v trifluoroacetic acid in water and *mobile phase* (B) comprising of acetonitrile. A linear gradient was programmed as described in Table 1. The optimum wavelength of 266 nm represents the wavelength of sufficient absorbance of all three drugs and was selected in order to permit the simultaneous determination
in FDCs. Mobile phase was pumped at a flow rate of 0.4 mL/ min.

Table1. *Mobile phase program for gradient elution*

Time (min)	Flow (ml/min)	Solvent A (%)	Solvent B (%)
0	0.4	95	05
1.0	0.4	95	05
2.0	0.4	85	15
6.0	0.4	85	15

Solvent (A): 0.1 %, v/v trifluoroacetic acid in water; solvent (B): acetonitrile.

Method validation

Linearity was established by triplicate injections of solutions containing standard AZT, 3TC and NVP in the concentration ranges of 100- 500, 50- 250 and 100- 180 mg/mL, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) values were calculated from the calibration curves as k SD/b where k = 3 for LOD and k = 10 for LOQ, SD is the standard deviation of the responses, and b is the slope of the calibration curve[9]. The intraday precision was established by making 3 injections of lowest, middle, and highest concentrations in the above ranges for AZT (100, 300, and 500 mg/mL) , 3TC (50, 150, and 250 mg/mL), and NVP (100, 140, and 180 mg/mL) on the same day. These injections were also repeated on 3 different days to determine interday precision. Accuracy was evaluated by recovery studies with 3 standard solutions containing known concentrations of AZT (240, 300, and 360 mg/mL), 3TC (120, 150, and 180 mg/mL) and NVP (160, 200, and 240 mg/mL), and the percent recoveries of the added drugs were determined. The specificity of the method was established through study of resolution factors of the drug peaks from the nearest peak, and also from all other peaks. The specificity of the method toward the drugs was also established through determination of the purity of AZT, 3TC and NVP peaks from UPLC analysis by using a PDA detector.

Robustness of the method was determined by deliberately varying certain parameters like flow rate (mL/min), concentration of acetonitrile (mL) in the mobile phase. One factor at a time was changed to estimate the effect. The assay was carried out in triplicate (n = 3) at a concentration of 200 mg/mL for AZT, 100 mg/mL for 3TC and 130 mg/mL for NVP. In the system suitability tests 3 replicate injections of freshly prepared working standard solutions of AZT (200 mg/mL), 3TC (100 mg/mL)and NVP (130 mg/mL) and 2 injections of the solutions prepared for the specificity procedure were injected into the chromatograph, and the relative standard deviation (RSD) values of the peak areas, resolution factors, tailing factors, and number of theoretical plates were determined.

Assay procedure

Drug contents were calculated by comparison with the appropriate standard solution of the drug. No

interferences due to excipients was detected in the spectra or chromatograms produced.

RESULTS AND DISCUSSION

Working conditions for the UPLC method were established with AZT, 3TC and NVP in bulk drugs and then applied to the pharmaceutical dosage forms. In order to affect the simultaneous analysis of three component peaks, an isocratic method was difficult. Hence, a gradient method was established since the chromatographic peaks were better defined and resolved. Under the experimental conditions investigated, the retention times for 3TC, AZT and NVP were 1.888, 3.497 and 4.786 min respectively, (Figure 2). The proposed method was found to be selective for the estimation of three drugs as recoveries of the drugs were not affected by the excipients and the excipient blend did not show any absorption in the range of analysis.

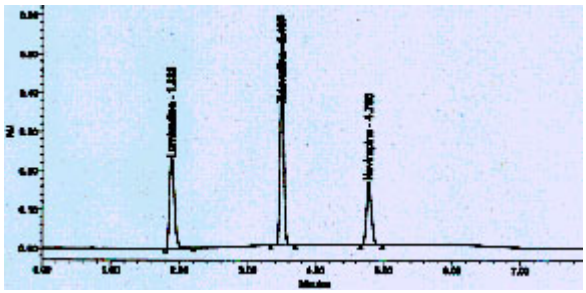

Fig.2 : *A representative UPLC chromatogram of AZT, 3TC and NVP (standard)*

Validation of the Proposed Method

The method was validated with respect to the following parameters given below as per ICH guidelines[10].

Linearity

Linear calibration plots for the above method were obtained in the calibration ranges of 100- 500, 50- 250 and 100- 180 mg/mL for AZT, 3TC and NVP, respectively, and the correlation coefficients obtained were > 0.999. The results show a good correlation between peak area and analyte concentration. The standard calibration curve was generated using regression analysis with Microsoft excel.

LOD and LOQ

The Limit of Detection (LOD) values for AZT, 3TC and NVP were observed 0.2792, 0.0676 and 0.3599 mg/mL, respectively, and the Limit of Quantitation (LOQ) values for AZT, 3TC and NVP were obtained 0.8462, 0.2050 and 1.0907 mg/mL, respectively.

Precision

Precision was studied to find out intra and inter day variations in the test method. The RSD values obtained were well below 2 % (i.e., in the ranges of 0.482- 1.162 and 0.432-1.569% for intra and inter day precision, respectively). The RSD values indicate that the method is sufficiently precise.

Accuracy

Recovery studies of the drugs were carried out for the accuracy parameter. These studies were carried out at three different concentration levels i.e. multiple level recovery studies. A known amount of AZT, 3TC and NVP standard were added into pre-analysed sample and subjected them to the proposed UPLC method. Percentage recovery was found to be within the limits.

Specificity

Specificity was checked by subjecting the drug solution in different stress conditions like Acid, Base, Peroxide and the degradation was noted. No degradation peak was observed.

Robustness

Robustness was done by small changing in the chromatographic conditions and found to be unaffected by small changing like ± 2% change in volume of organic solvents of mobile phase. The method remained robust even with small variations in temperature (± 0.5°C). There was no significant difference in peak area and retention time. It was also found that acetonitrile from a different manufacturer had no significant influence on the determination. Insignificant differences in peak areas and less variability in retention times were observed.

System suitability

The parameters, retention time, resolution factor, tailing factor, and number of theoretical plates were evaluated. The results given in Table 2 shows the variation in retention times among 3 replicate injections of AZT, 3TC and NVP standard solutions was very low, with RSD values of 0.2, 0.2 and 0.4 %, respectively.

Table 2. *System suitability parameters for the determination of zidovudine (AZT), lamivudine (3TC) and nevirapine (NVP) by the proposed UPLC method*

Parameter	3TC	AZT	NVP
RT ±SD, min	1.888 ± 0.013	3.497 ± 0.015	4.786 ± 0.013
USP Resolution	-	14.23	11.29
USP Tailing factor ±SD	1.36 ± 0.018	1.23 ± 0.015	1.19 ± 0.017
USP Plate Count ±SD	3357 ± 0.197	22243 ± 0.285	21092 ± 0.296

Assay of AZT, 3TC and NVP in tablets

The assay results obtained are 101.1 ±0.2 %, 99.87 ±0.2 % and 99.89 ±0.2 % for zidovudine, lamivudine and nevirapine, respectively.

CONCLUSION

The proposed method gives a good resolution between three drugs AZT, 3TC and NVP. It can be concluded that the method is sufficiently specific and reproducible in the analysis of three drugs with good resolution. All the parameters for three drugs met the criteria of ICH guidelines for method validation. The developed method may therefore be recommended for routine quality control analysis of the investigated drugs.

ACKNOWLEDGEMENTS

The authors are thankful to the Waters India Ltd (Noida, India) for providing the excellent facilities for carrying out this research work. Thanks are also extended to Aurobindo Pharma Ltd (Hyderabad, India) for providing standard and samples of AZT, 3TC and NVP.

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