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RP-HPLC METHOD DEVELOPMENT AND METHOD VALIDATION OF SIMULTANEOUS ESTIMATION OF ZIDOVUDINE LAMIVUDINE NEVIRAPINE IN FIXED DOSE COMBINATION

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ABSTRACT

The present research work was carried out using a simple RP-HPLC method for simultaneous estimation of zidovudine, lamivudine and nevirapine in bulk and pharmaceutical formulation. The separation was carried out by Phenomenox C18 (4.6 ×250mm, 5µ ID) used as a stationary phase and MeCN: MeOH and 0.1% of DEA (pH 3.0 was adjusted with 10% OPA) used as a mobile phase in the ratio of 30:15:55 at a 0.9ml^{-1} flow rate and the peak detection was carried out 245nm (isobestic point). Overall run time was 10.0 min and the retention time was 2.49, 3.98, 7.01 min for zidovudine, lamivudine and nevirapine respectively. The proposed method was validated as per the ICH guidelines and found to be specific, linear, selective and precise. This method can be applied on bulk and commercially available individual and combined pharmaceutical dosage forms.

KEYWORDS

Zidovudine, Lamivudine, Nevirapine, RP-HPLC and Simultaneous estimation.

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INTRODUCTON

The fixed dose combination is highly efficient in eradicating human immune deficiency virus (HIV), since single drug therapy is rapidly ineffective for antiretroviral treatment because of its drug resistance; and now a day's new paradigm is to combine more than two combinations are preferred for antiretroviral therapy. Zidovudine (AZT), Lamivudine (LMV) and Nevirapine (NVP) are a nucleoside reverse transcriptase inhibitors (NRTI) were officially approved for treating against antiretroviral therapy [Herve *et al*, 2007[1], Stefania *et al*, 2008; Luisa *et al*, 2002[2]]. AZT is the first officially USFDA approved drug for the treatment of HIV and LMV was approved for combination

with other retroviral drugs and its special socioeconomic importance because of their widespread frequency in humans. LMV is a synthetic dideoxy- nucleoside derivative that is active against HIV and hepatitis B virus (HBV) and NVP is an inhibitor of DNA and RNA dependent DNA polymerase effectively inhibits the HIV-I. The chemical structure of Zidovudine, Lamivudine and Nevirapine was given in Figure No.1.

AZT is official in pharmacopeias (IP, BP, USP, EP). Several methods have been cited in the literature for the determination of AZT in pharmaceutical formulation [Bengi U and Sibel A.H, 2002[3], Adams *et al*, 2009[4]] and in biological fluids by LC [Soumya *et al*, 2006, Yihui Deng *et al*, 2007[5]]. The quantitative determination of AZT and metabolites in cell extracts by LC with solid-phase extraction [Lefebvre *et al*, 2007[6]], micellar electrokinetic method [Steward and Bin fan, 2002[7]] were also reported.

LMV is official in pharmacopeias [IP, USP, BP, EP]. Several HPLC methods have been cited in the literature for the individual estimation of LMV in human plasma [Joanna *et al*, 2001[8]] in saliva and cerebrospinal fluid [Hoetelmans *et al*, 1998[9]]. A simultaneous HPLC estimation of LMV and Stavudine in pharmaceutical formulation is described [Jayaraman *et al*, 2012[10]], Another HPLC method has been reported for the simultaneous estimation of LMV along with Stavudine and Nevirapine [Ramesh *et al*, 2006[11], Ramyakrishnan *et al*, 2013[12]]. Simultaneous estimation of LMV and Zidovudine by LC-MS in human plasma [Tidwell *et al*, 2000[13], Poirier *et al*, 2000] and simultaneous estimation of LMV, Zidovudine and lamivudine in human plasma by LC-MS-MS method [Robbins *et al.,* 2007[14]] also reported. Further a stability indicating HPLC assay method is proposed for the simultaneous estimation of LMV and AZT in formulation [Santoshkumar and venkateshwarrao, 2014[15]].

NVP is official in pharmacopeias [IP, BP, USP, EP]. Various methods have been reported for the simultaneous estimation of NVP by HPLC [Emilia Marchei, *et al*, 2002[2], Vibhutikabra *et al*, 2009[16], Vamshi Krishna *et al*, 2012[17], Ramaprasad *et al*, 2012[18], by ion-pair chromatographic method [Stewart and Bin Fan, 2002[7]], by LC-MS-MS method [Li-Jun *et al*, 2010[19]]. Although there are number of methods reported for the analysis of AZT, LMV and NVP individually and in combination with other drugs, no method is found available for the simultaneous estimation of all these drugs with single mobile phase system.

EXPERIMENTAL

MATERIAL AND METHODS

Working standards of Zidovudine (AZT), Lamivudine (LMV) and Nevirapine (NVP) weregifts from Hetro Labs., Hydrabad, India. Themarketed formulation was purchased from whole saler (LAZID-N tab). Acetonitrile (MeCN) and Methanol (MeOH) of HPLC grade and Triethylamine (TEA), Diethylamine (DEA) and other reagents of analytical-reagentgrade were from SD Fine Chemicals (Mumbai, India). The HPLC grade water was prepared by using Milli-Q Academic, Millipore, Bangalore, India.

HPLC instrumentation and conditions

Chromatographic separations of AZT, LMV and NVP were carried out, with the ternary mobile phase consisted of a mixture of MeCN: MeOH and 0.1% of DEA (pH 3.0 was adjusted with 10% ortho phosphoric acid) and Phenomenox C18, (4.6 ×250mm, 5µ ID)was used as a stationary phase then detection were made at 245nm at ambient temperature.

Stock and working standard solutions

Stock and working standard solutions of AZT, LMV, NVP were prepared using mobile phase as a diluting solvent. Standard solutions employed for the optimization procedure constituted a mixture of AZT, LMV, NVP at10.0, 10.0 and 10.0µgmL^{-1} were prepared respectively. For quantification of analytes in markets formulation samples, individual calibration curves (peak area ratios of AZT, LMV, NVP versus drug concentrations) were established at five levels; 2.0-10µg mL^{-1} for LMV and 1.0- 5.0 for AZT and NVP.

Formulation sample preparation

Twenty tablets were weighed and finely powdered. In the case of capsule dosage, the contents of the capsule were mixed thoroughly. An amount of pharmaceutical products powder equivalent to 50mg of AZT, LMV, NVP was accurately weighed and transferred in a 50ml volumetric flask. This

mixture was sonicated for 15 min for complete extraction of drugs and the solution was made up to the mark with the mobile phase. Then further diluted, to obtain a concentration of 10µg/ml for AZT, NVP and LMV, respectively. The solution was centrifuged at 5000RPM for 15 min; the clear supernatant was collected and filtered through a 0.2µm membrane filter (Gelman Science, India) and 20µl of this solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Preliminary experiments

Initial studies were carried out from the literature review by trial and error method to identify the basic requirements of liquid chromatographic method developments such as (i) type of stationary phase (C18, C8 and C6), (ii) range of pH, (iii) flow rate (iv) type of mobile phase additives (Diethylamine, Triethylamine, THF), based on the studies. To obtain an acceptable analytical retention time, good quality of separation (resolution, capacity factor), there is need to optimize the chromatographic separation. For the optimization purpose we further studies conducted.

Selection of stationary phase

There are different types of stationary phase available for the reverse phase HPLC and we tried phenyl, C18, C8 and C6 columns. Well resolved peak separation and excesses of asymmetric factor, less peak resolution were observed on C8 and C6 columns. Moreover phenyl columns are not suitable for this analyte. Among these C18 gave good peak separation and satisfactory retention time, resolution and capacity factor.

Selection of mobile phase

Initially acetonitrile was selected as the organic phase and HPLC water was selected as an aqueous phase, then various ranges of pH (pH was adjusted with 10% orthophosphoric acid) were tried. In the above combination of mobile phase were tested in different proportion (50:50, 40:60, 60:40, 70:30) and at 50: 50 (MeCN: water (pH 3.5) ratio only we observed valuable retention time but poor resolution, capacity factor and poor peak separation, then introduced methanol to overcome this problem.

Selection of Additives

From the selected above mobile phase we added 0.05 to 1.0% triethylamine and there are no significant changes in resolution, and the peak overlapping. Then we tried acetic acid (0.1- 0.5%) in aqoues phase small variation in resolution, so then tried with 0.05 - 1.0% of diethylamine, it produced significant improvements in resolution and good peak shape.

Method Validation

The proposed liquid chromatographic method was validated by following ICH guidelines. Validation parameters like selectivity, specificity, linearity, limit of detection and quantification, accuracy, precision, stability and robustness were addressed.

Specificity

The specificity of the method was evaluated by assessing the chromatograms of most commonly used excipients (starch, lactosemonohydrate, methyl cellulose, titanium dioxide and magnesiumstearate) with that of the standard drugs. There were no excipient speaks co-eluted with the analytes, indicating that the optimized assay method is selective and specific in relation to the excipients used in this study.

Linearity

The linearity of the method was established at five levels over the concentration ranges of 2.0-10µg mL^{-1} for LMV and 1.0- 5.0 for AZT and NVP approximately from 20 to 200% of nominal range of analyte. Peak areas (y) of LMV and for AZT and NVP were plotted versus their respective concentrations (χ) and linear regression analysis performed on the resultant calibration curves (n=6). The correlation coefficients (R2) were found to be more than 0.999 and the details given in Table No.1 and Figure No.2.

Limits of Detection (LOD) and Limits of Quantitation (LOQ)

In accordance with ICH recommendations, the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/ (slope of the regression equation)] by multiplying with 3.3 and 10 respectively. Using the above equations, the LOD and LOQ were estimated at4.42ng mL-1 and

12.41ng mL-1 for LMV, 1.06ng mL-1 and 4.31ng mL-1 for NVP, 2.32ng mL-1 and 7.43ng mL-1 for AZT, respectively.

Accuracy

The accuracy of the method was determined by analyzing Quality Control (QC) standards prepared at three levels of 80, 100 and 120% of the expected assay value or label claim of the analytes in the commercial formulation. QC samples were prepared as three replicates at each concentration level by spiking the standard drugs with the placebo excipients, which were left overnight to allow matrix-analyte interactions to occur. The %recovery of the analytes at each level (n = 3) and mean % recovery (n = 9) were determined and % accuracy was expressed as [(calculated amount/predicted amount) × 100]. Accuracy, assessed by spike recovery, in which the % recovery of both enantiomers it is at each level (n = 3) and mean % recovery

(n = 9) were found to be 99.68, 99.72 and 99.72% for LMV, AZT and NVP respectively. The recoveries of each drugsat each level were found well within the acceptable criteria of bias, \ ±2.0%. The mean % recovery (n = 9) for each enantiomer was also tested for significance by using Student t-test. Since the tCalc is less than the theoretical t value (tCrit = 2.306), at 5% significance level, the null hypothesis (the recovery is unity or 100%) was accepted.

Precision

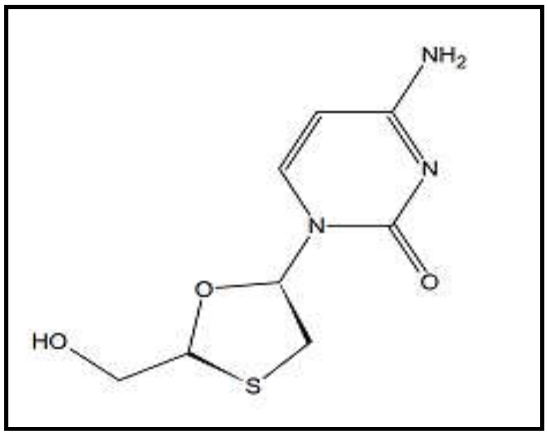
The precision was established by injecting three different concentrations of each analyte for LMV, AZT and NVP each in six replicates, for intraday precision (repeatability) and on three consecutive days for the intermediate precision (reproducibility). Precision was expressed by the %RSD of the analyte peak area. Results for all studied compounds met the proposed requirement %RSD ≤ 2%. The intra and inter-day precision (n = 6.0) was confirmed since, the % CV were well within the target criterion of ≤ 2.0.

Robustness

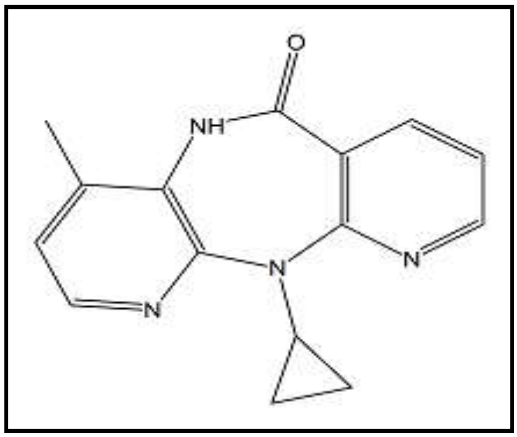
The robustness of the proposed method was assessed to provide an indication of its reliability during normal usage with respect to small, but deliberate variations in experimental parameters such as variations in MeCN concentration (30% ± 0.5), the flow rate (0.9 ± 0.05) and the pH (3.0 ± 0.5%) did not alter the assay values more than 1.0% and therefore it would be concluded that the method conditions are robust.

Table No.1: Validation parameters of zidovudine, lamivudine, nevirapine

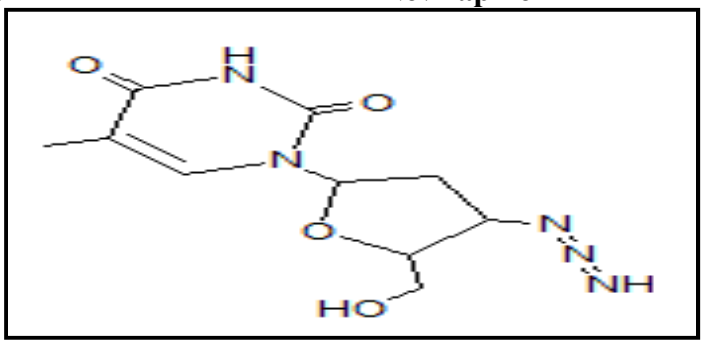
S.No	Parameters	LMV	AZT	NVP
1	Linearity range (µg/ml)	2-10µg/ml	1-5µg/ml	1-5µg/ml
2	Slope	423416x	951195x	846832x
3	Correlation coefficient R2	0.999	0.9993	0.999
4	Rt	2.49 min	3.98 min	7.01 min
5	Tailing factor	0.6	0.4	1.1
6	LOD	4.42ng/ml	1.06ng/ml	2.32ng/ml
7	LOQ	12.41ng/ml	4.31ng/ml	7.43ng/ml
8	Theoretical plates (USP)	4207	4550	5485



Lamivudine

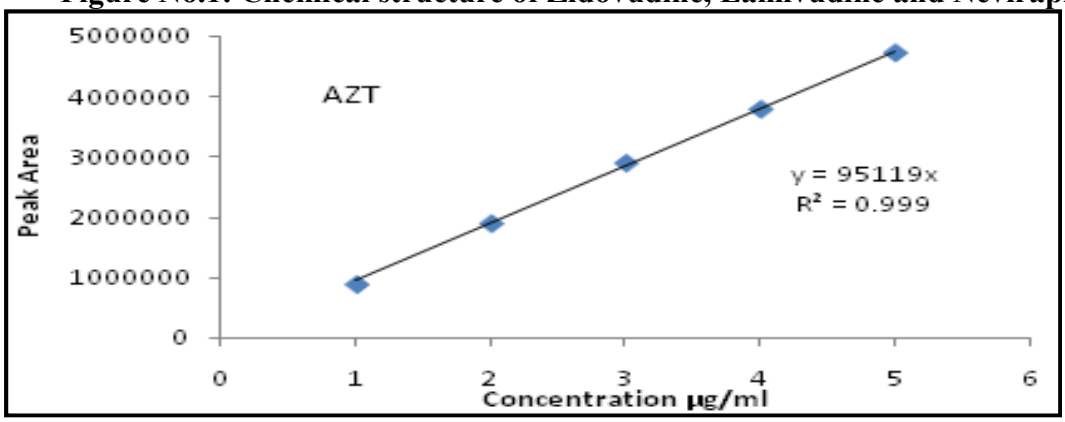


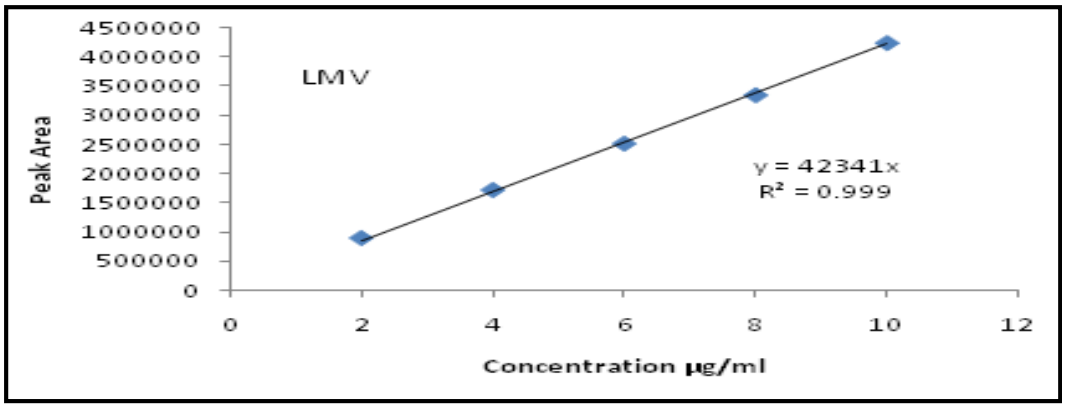
Nevirapine



Zidovudine

Figure No.1: Chemical structure of Zidovudine, Lamivudine and Nevirapine





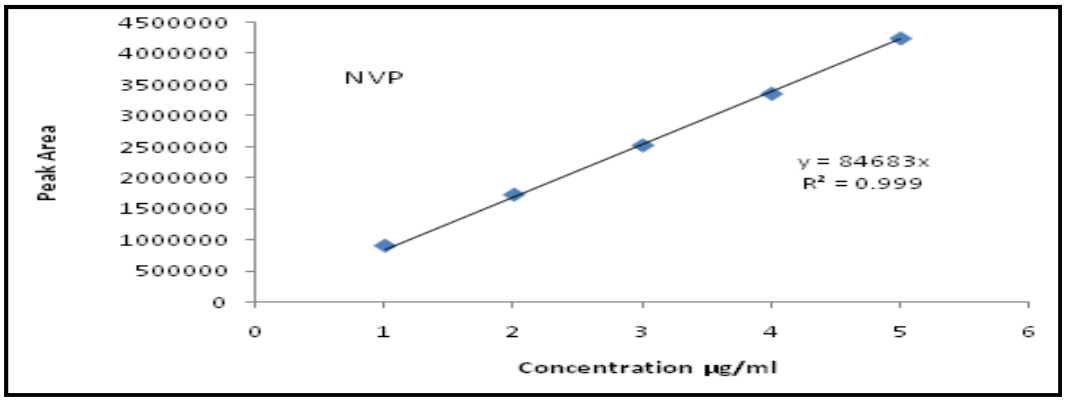


Figure No.2: Linearity graph of zidovudine, lamivudine, nevirapine

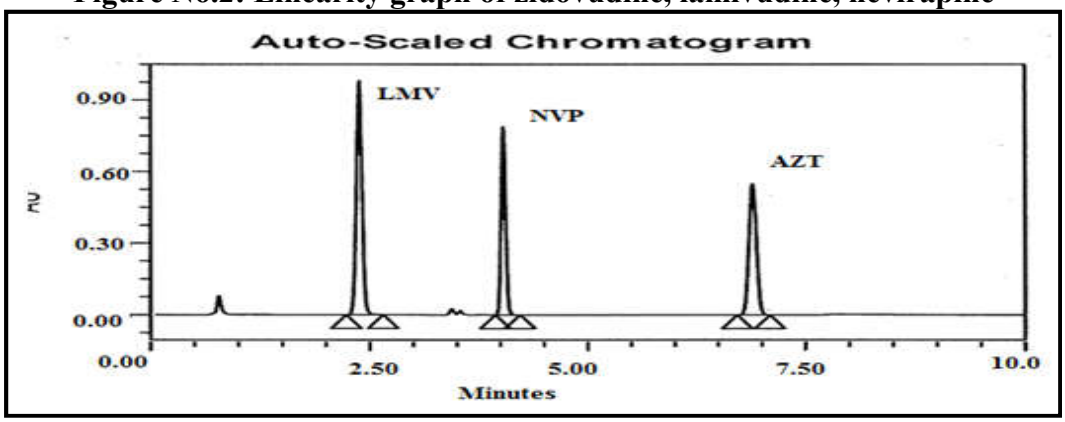


Figure No.3: Respective chromatograms of LMV, NVP, AZT optimal conditions [LMV (10.0µg mL^{-1}), NVP (5.0µg mL^{-1}), AZT (5.0µg mL^{-1})]

CONCLUSION

A rapid, simple, robust and efficient isocratic reversed-phase high-performance liquid chromatography method was developed, optimized and validated for the simultaneous determination of the LMV, NVP and AZT, in pharmaceutical formulations.

The analytical results obtained lead to the conclusion that the developed method performs well with regard to precision, accuracy, rapidity, sensitivity and robustness, with single mobile phase allows to detect LMV, NVP and AZT. Therefore, it could be successfully employed for the analysis of these antiretroviral drugs in formulations samples.

This optimized method has to be utilized for the simultaneous quantitative analysis of AZT, LMV, NVP, and STV in pharmaceutical formulation and biological matrix. The method can be applied for the marketed (commercial) formulation samples such as LAZID-N tab containing (AZT= 300mg, LMV= 150mg, NVP= 200mg). The mean, % SD recoveries values achieved were within the parenthesis being the % CV of the six replicates and the % CV of the assay results were < 2.0, indicating the precision of the analytical methodology.

The mean recoveries for each analyte were also tested for significance to realize whether the recovery means are different from the label claim of the tablet by employing student *t*-test. The values of t_{Calc} for AZT, LMV, and NVP were obtained less than the t_{Crit}= 2.430 at 5.0% significance level, suggested that there was no significant difference within the mean recoveries of the analytes and the label claim of the analyzed commercial formulation.

CONFLICT OF INTEREST

All authors' declared no conflict of interests.

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