International Journal for Advanced Review and Research in Pharmacy (IJARRP)

Stability Indicating RP - HPLC Method Development and Estimation of Zidovudine Tablet

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Abstract:
A simple, precise, accurate, and rapid reverse phase-high performance liquid chromatography (RP-HPLC) method with UV-Visible detector has been developed and estimation of Zidovudine tablet dosage form.

Keywords:
Zidovudine, RP-HPLC, Method Development

GENERAL METHODOLOGY FOR THE DEVELOPMENT OF NEW HPLC METHODS

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

1. Careful sampling and sample preparation.
2. Precise sample injection.
3. Appropriate choice of the column.
4. Choice of the operating conditions to obtain the adequate resolution of the mixture.
5. Reliable performance of the recording and data handling systems.
6. Suitable integration/peak height measurement technique.
7. The mode of calculation best suited for the purpose
8. Validation of the developed method.

a) Careful sampling and sample preparation

Before beginning method development, it is need to review what is known about the sample in order to define the goals of separation[1].

- Number of compounds present
- Chemical structures (functionality of compounds)
- Molecular weights of compounds
- pKa values of compounds
- UV spectra of compounds
- Concentration range of compounds in samples of interest
- Sample solubility

b) Separation goals1[2]

The goals of HPLC separation need to be specified clearly include

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis
- It may be necessary to separate all degradants or impurities from a product for reliable content assay or not
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of ± 1 to 2% is usually achievable)
- Whether a single HPLC procedure is sufficient for raw material or one or more for mutations and/or different procedures are desired for formulations
- When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important.

c) Sample preparation

Samples come in various forms

- Solutions ready for injections
- Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- Solids that must first be dissolved or extracted
- Samples that require pretreatment to remove interferences and/or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate "column killers".

In many cases the development of an adequate

sample pretreatment can be challenging than achieving a good HPLC separation.

The detector selected should sense all sample components of interest. Variable-wavelength ultraviolet (UV) detectors normally are the first choice, because of their convenience and applicability for most samples. For this reason, information on the UV spectra can be an important aid for method development. When the UV response of the sample is inadequate, other detectors are available (fluorescence, electrochemical etc) or the sample can be derivatized for enhanced detection[3].

d) Precise sample injection

Before the first sample is injected during HPLC method development, we must be reasonably sure that the detector selected will sense all sample components of interest.

Variable wavelength UV detectors normally are the first choice, because of their convenience and applicability for most samples. Information on the UV spectra can be an important aid for method development[4].

e) Choice of the Column

The selection of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size etc of the ions generated by the sample molecules and the nature of ionizable group on the stationary phase.

Table No. 01: Selection of columns based on the method is briefly summarized in the table below

Method/ Description/ Columns	Preferred Method
Reverse-phase HPLC	
Uses water- organic mobile phase Columns: C18 (ODS), C8, phenyl, trimethylsilyl (TMS), cyano.	First choice for most samples, especially neutral or non-ionized compounds that dissolve in water-organic mixtures
Ion-pair HPLC	
Uses water-organic mobile phase, a buffer to control pH, and an ion-pair reagent Columns: C18, C8, Cyano	Acceptable choice for ionic or ionisable compounds, especially bases or cations.
Normal-phase HPLC	
Uses mixtures of organic solvents as mobile phase Columns: cyano, diol, amino, silica	Good second choice when reverse-phase or ion-pair HPLC is ineffective; first choice for lipophilic samples that do not dissolve well in water-organic mixtures; first choice for mixtures of isomers and for preparative HPLC

g) Choice of the operating conditions to obtain the adequate resolution of the mixture

If HPLC is chosen for the separation, the next step is to classify the sample as regular or special.

Regular samples mean typical mixtures of small molecules (<2000Da) that can be separated using more or less standardized starting conditions.

Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reverse phase columns are recommended.

Table No. 02: Preferred experimental conditions for the initial HPLC separation

Separation Variable	Preferred Initial Choice
Column	
Dimensions (length, ID)	15 X 0.46 cm
Particle size	5 mm
Stationary phase	C8 or C18
Mobile phase	
Solvents A and B	Buffer- acetonitrile
% B	80-100%
Buffer (compound, pH, concentration)	25mM potassium phosphate, 2.0<pH<3.0
Additives (e.g., amine modifiers, ion- pair reagents)	Do not use initially
Flow rate	1.5-2.0 mL/min
Temperature	35-45oC
Sample Size	
Volume	< 25mL

Polar solvent

a. 3.5 mm particles are an alternative, using a 7.5 cm column.

b. For an initial isocratic run; an initial gradient run is preferred.

c. No buffer required for neutral samples; for pH <2.5, pH-stable columns are recommended.
d. Smaller values required for smaller-volume columns (e.g., 7.5 x 0.46-cm, 3.5-mm column).

Using these conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable.

If typical reverse-phase conditions provided cause is inadequate sample retention, suggesting the use of either ion-pair or normal phase HPLC. Alternatively, the sample may be strongly retained with 100% ACN as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC. (15)

i) Getting Started On Method Development

One approach is to use an isocratic mobile phase of some average solvent strength (50%) organic solvent. A better alternative is to use a very strong mobile phase first (80-100%) then reduce %B as necessary. The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity.

Table No. 03: Goals that are to be achieved in method development

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that Rs be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	≤2% (1 SD) for assays; ≤5% for less-demanding analyses ≤15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Roughly in order of decreasing importance but may vary with analysis requirements.

Separation or resolution is a primary requirement in quantitative HPLC. The resolution (Rs) value should be maximum (Rs > 1.5) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions.

Therefore, values of Rs=2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and greater method ruggedness.

Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis). In such cases only enough separation of individual components is required to provide characteristic retention times for peak identification.

The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable).

Conditions for the final HPLC method should be selected so that the operating pressure with a new column does not exceed 170 bar (2500 psi) and an upper pressure limit below 2000 psi is desirable. There are two reasons for this pressure limit, despite the fact that most HPLC equipment can be operated at much higher pressures. First, during the life of a column, the backpressure may rise by a factor of as much as 2 due to the gradual plugging of the column by particulate matter. Second, at lower pressures (<170 bars, pumps, sample valves and especially auto samplers operate much better, seals last longer, columns tend to plug less and system reliability is significantly improved. For these reasons, a target pressure of less than 50 % of the maximum capability of the pump is desirable. When dealing with more challenging samples or if the goals of separation are particularly stringent, a large number of method development runs may be required to achieve acceptable separation. (16)

k) Repeatable separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and temperature) between method development experiments, enough time must elapse for the column to come into equilibrium with the new mobile phase and temperature.

Usually column equilibration is achieved after passage of 10 to 20 column volumes of the new mobile phase through the column. However, this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back-to-back repeat experiments (± 0.5% or better), it can be assumed that the column is equilibrated and the experiments are re-

peatable.

l) Completing the HPLC method development

The final procedure should meet all the goals that were defined at the beginning of method development. The method should also be robust in routine operation and usable by all laboratories and personnel for which it is intended. (17)

Parameters of HPLC method

System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed.

The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation (SD) can be determined during validation.

System suitability might then require that retention times fall within a ± 3 SD range during routine performance of the method.

The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. These parameters include plate number (N), tailing factor, k and or a, resolution (Rs) and relative standard deviation (RSD) of peak height or peak area for respective injections.

Retention Time (Rt)

The time between the injection point and the maximum detector response for correspondent compound.

Retention Volume (Vr)

t_Rx eluent flow rate

Theoretical Plates

The column performance can be defined in terms of column plate number 'n' is defined by

n = 5.54 (tR/W½)2

Where 't_R' is the retention time of the peak and
'W½' is the width of the peak at half peak height.

HETP

Another way to express efficiency of column is by calculating height equivalents of theoretical plates (HETP).

h = L/n

Where h = HETP;
L = Length of column;
n = number of theoretical plates. Lower the HETP, higher is the efficiency of the column, i.e., higher the theoretical plates more efficient the column is.

Resolution

The resolution of two adjacent peaks can be calculated by using the formula

Rs = 1.18(t2-t1) / W0.5, 1 + W0.5, 2

Where t1 and t2 are retention times of the adjacent peaks and
W0.5,1 and W0.5,2 are the width of the peaks at half height.
Rs = 2.0 or greater is a desirable target for method development.

Retention Factor

The retention factor k is given by the equation

k = (tR – t0)/ t0

Where tR is the band retention time and t0 is the column dead time.

Peak Symmetry

The peak symmetry can be represented in terms of peak asymmetry factor and peak tailing factor which can be calculated by using the following formulae

Peak asymmetry factor = B/A

Where 'B' is the distance at 50% peak height between leading edge to the perpendicular drawn from the peak maxima and 'A' is the width of the peak at half the peak height.

Peak Tailing Factor

According to USP (2000) Peak tailing factor can be calculated by using the formula

T = W0.05 / 2f

Where W0.05 is the width of the peak at 5 % height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 50 % of the peak height from the base line.

EQUIPMENTS AND CHEMICALS

A simple reverse phase HPLC method was developed for the determination of present in zidovudine pharmaceutical dosage form of 400mg. column used kromocil(150*4.6µm packed with 5µm) in an isocratic mode with mobile phase Buffer: Methanol (60:40) was used. The flow rate was 1.3ml/ min and effluent was monitored at 266 nm and column temperature of 250C.

Equipment and Apparatus used:

1. HPLC with PDA detector (Waters)
2. Sonicator (Ultrasonic sonicator)
3. PH meter (Thermo scientific)
4. Micro balance (Sartorius)
5. Vacuum filter pump

Reagents used:

1. Methanol HPLC Grade (RANKEM)
2. Acetonitrile HPLC Grade (RANKEM)
3. HPLC grade Water (RANKEM)
4. Glacial Acetic acid

METHOD DEVELOPMENT

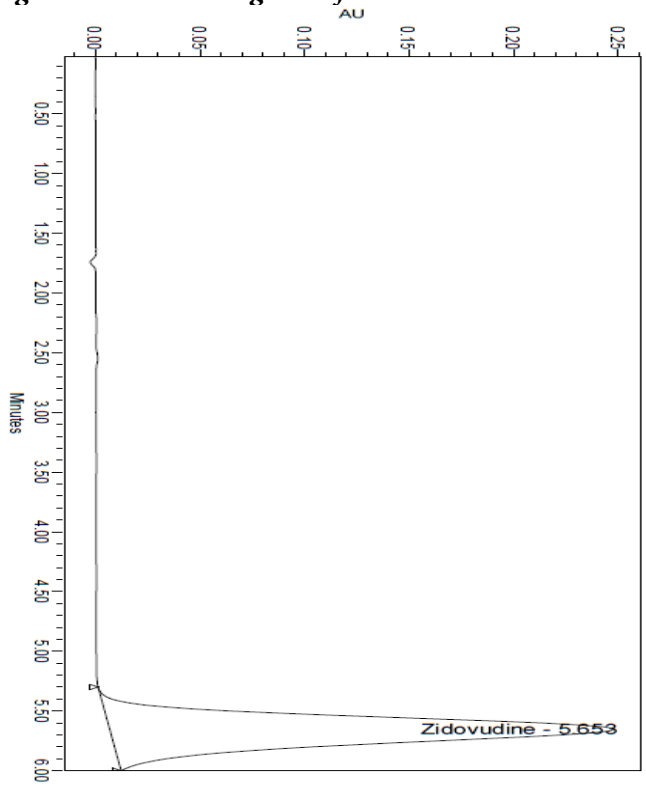
Based on drug solubility and Pka Value following conditions has been used to develop the method estimation of Zidovudine.

Trial 1

Chromatographic Conditions

Column	:	Kromocil-(150*4.6 µm)
Mobile phase	:	Buffer: Methanol (80:20)
Buffer	:	0.02M Sodium acetate PH 5.5 adjusted with Glacial acetic acid
Flow rate	:	1.0 ml/min
Detector	:	UV at 266nm
Run time	:	20 minutes
Diluent	:	Water: Methanol (90:10)
Temperature	:	250C
Injection Volume	:	20µL

Fig. No. 01 Chromatogram of Trial 1

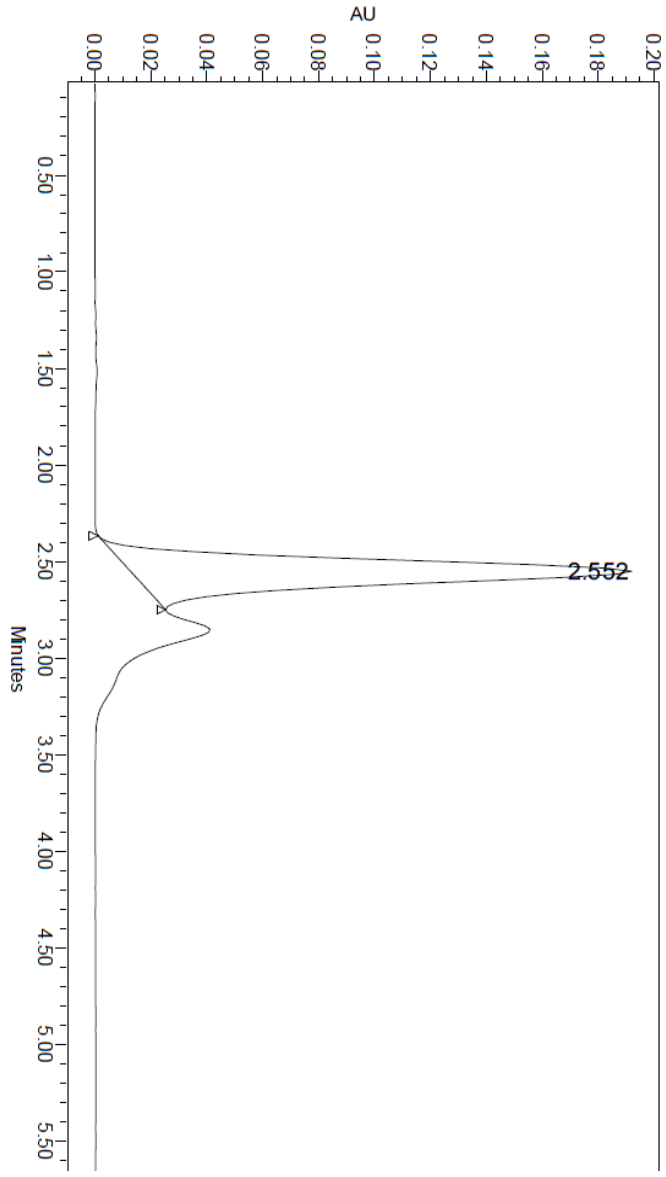


Observation: Peak shape was not good and retention was good.

Trial 2

Column	:	Kromocil-(150*4.6 µm)
Mobile phase	:	Buffer: Methanol (70:30)
Buffer	:	0.02M Sodium acetate PH 5.3 adjusted with Glacial acetic acid
Flow rate	:	1.3ml/min
Detector	:	UV at 266nm
Run time	:	20 minutes
Diluent	:	Methanol: Water (10:90)
Temperature	:	250
Injection Volume	:	20µL

Fig. No. 02 Chromatogram of Trial 2



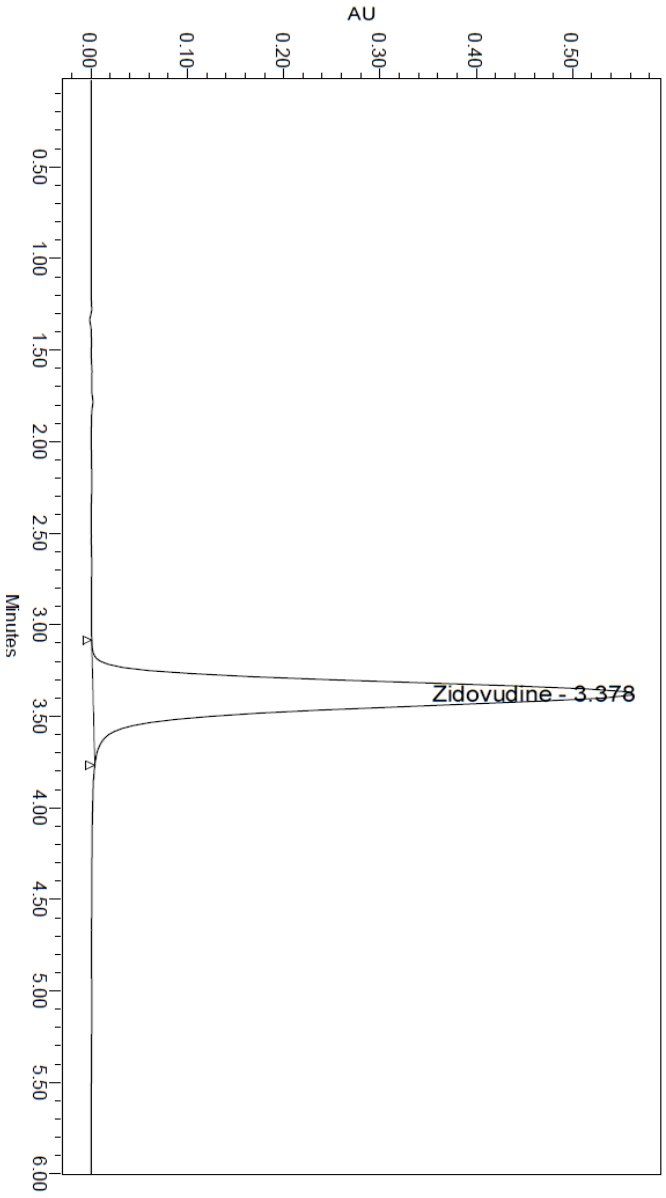
Observation:

Peak shape was improved but not up to the mark.

Trial 3

Column	:	Kromocil-(150*4.6 µm)
Mobile phase	:	Buffer: Methanol (60:40)
Buffer	:	0.02M Sodium acetate PH 5.3 adjusted with Glacial acetic acid
Flow rate	:	1.3ml/min
Detector	:	UV at 266nm
Run time	:	20 minutes
Diluent	:	Methanol: Water (10:90)
Temperature	:	250
Injection Volume	:	20µL

Fig. No. 03 Chromatogram of Trial 3



Observation: Peak shape was symmetrical and retention was also good.

Optimized method

Column	:	Symmetry C18, 250×4.6mm, 5µm or Equivalent
Mobile phase	:	Buffer: Methanol (60:40)
Buffer	:	0.02M Sodium acetate PH 5.5 adjusted with Glacial acetic acid
Flow rate	:	1.3ml/min
Detector	:	UV at266nm
Run time	:	20 minutes
Diluent	:	Methanol: Water (10:90)
Temperature	:	250C
Injection Volume	:	20µL

Preparation of Mobile phase:

Mobile Phase: Buffer: Methanol (60:40)

Buffer Preparation:

Dissolve 3gm of sodium acetate 900 mL of water and mix. Adjust the pH of this solution to 5.3 (±0.05) with glacial acetic acid.

Diluent: Methanol: Water (30:70)

Stock and Standard Solution Preparation:

Weigh accurately about 50mg Zidovudine working standard and transfer into a 50 mL volumetric flask, add 35 mL of diluent and sonicate to dissolve for about 5 min, further made up the volume with diluent (Stock Solution). From stock solution 2 ml was taken in 10 ml volumetric flask and volume was made up to the mark with diluent (Standard Solution).

Preparation of Linearity Solutions

Preparation of 50% Solution (100ppm):

From stock solution 1 ml was taken into the 10 ml volumetric flask and volume was made up with diluent

Preparation of 75% Solution (150ppm):

From stock solution 1.5 ml was taken into the 10 ml volumetric flask and volume was made up with diluent

Preparation of 100% Solution (200ppm):

From stock solution 2 ml was taken into the 10 ml volumetric flask and volume was made up with diluent

Preparation of 125% Solution (250ppm):

From stock solution 2.5 ml was taken into the 10 ml

volumetric flask and volume was made up with diluent

Preparation of 250% Solution (300ppm):

From stock solution 3 ml was taken into the 10 ml volumetric flask and volume was made up with diluent

Sample Preparation:

Weigh 20 tablets and crush into powder. Weigh powder equivalent to 500 mg of the Zidovudine and transfer into a 100 mL volumetric flask, add 70 mL of diluents and sonicate for 15 min , further make up the volume with diluent. Further dilute the filtrate 4 mL to 100 mL with diluent.

Assay Methodology

Assay of the marketed formulation was carried out by injecting sample corresponding to equivalent weight into HPLC system. And percent purity was found out by following formulae.

Calculate the percentage purity of Zidovudine present in tablet using the formula:

Calculation:

Assay =

Spl area Std. Dil. Fac Avg. Wt of Tab

--------- X---------- X ----------X Potency of

Std area Spl. Dil. Fac L.C Std

Spl area – Sample Peak area

Std area – Standard Peak area

SYSTEM SUITABILITY

A Standard solution of Zidovudine working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

Fig. No. 06 Chromatogram of Standard Injection-

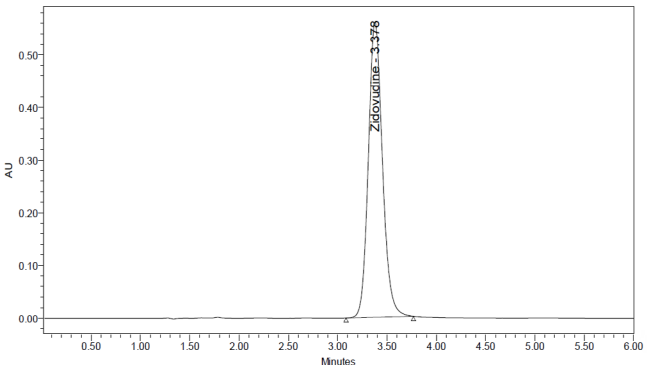


Fig. No. 07 Chromatogram of Standard Injection-2

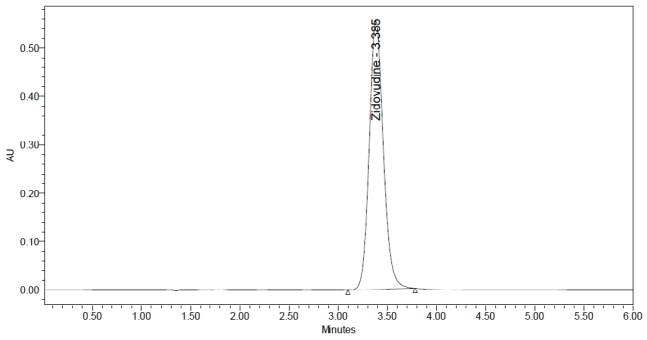


Fig .No. 08 Chromatogram of Standard Injection-3

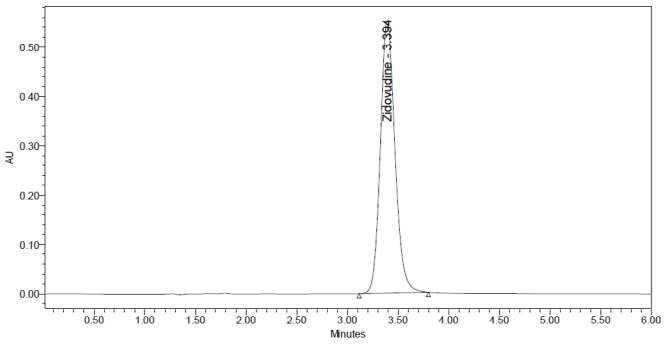


Fig. No. 09 Chromatogram of Standard Injection-4

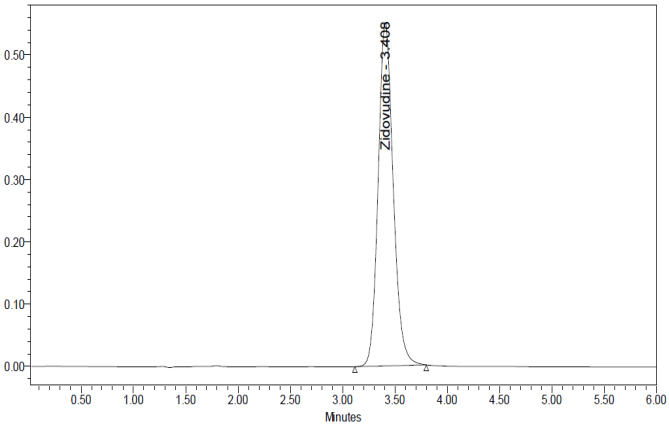
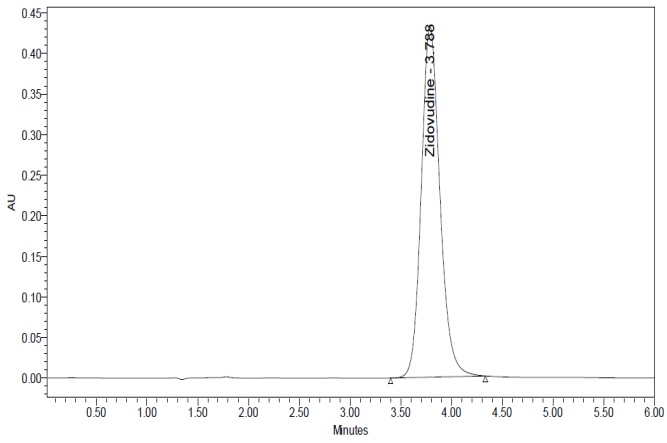


Fig .No. 10 Chromatogram of Standard Injection-5



LINEARITY:

To demonstrate the linearity of assay method, inject 5 standard solutions with concentrations of about 100 ppm to 300 ppm of Zidovudine. Plot a graph to concentration versus peak area. The results were summarised in table 1&2.

Fig. No. 11 Chromatogram of Linearity Concentration Level (50% or 100ppm)

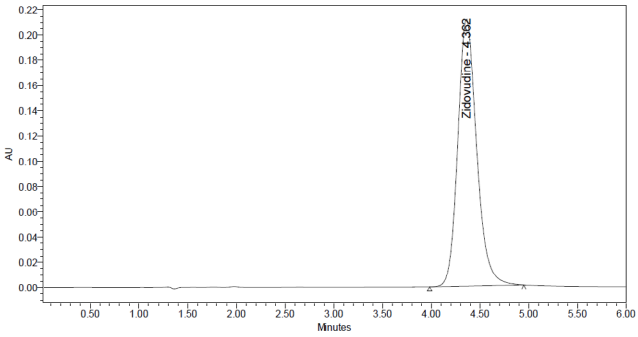


Fig. No. 12 Chromatogram of Linearity Concentration Level (75%, or 150ppm)

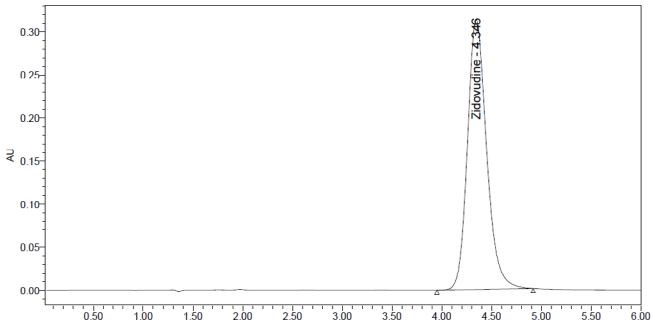


Fig. No. 13 Chromatogram of Linearity Concentration Level (100%, or 200ppm)

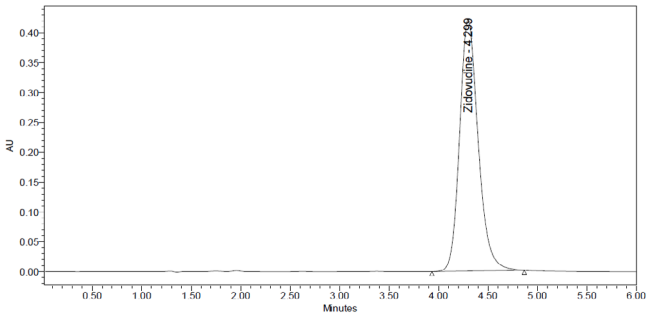


Fig. No. 14 Chromatogram of Linearity Concentration Level (125%, or 250ppm)

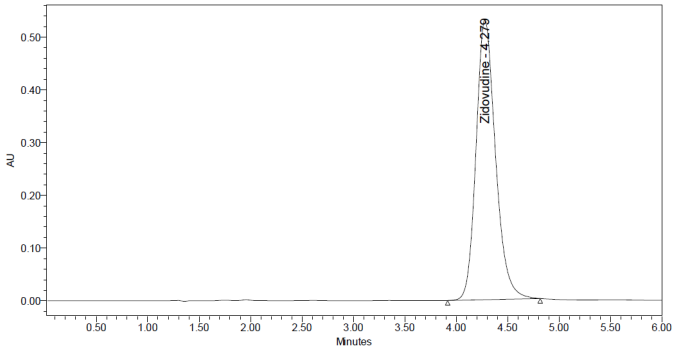
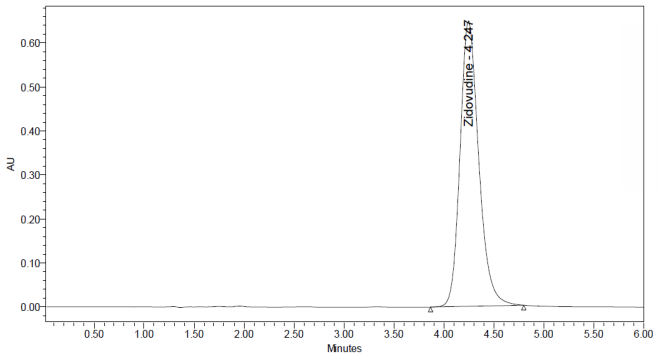


Fig. No. 15 Chromatogram of Linearity Concentration Level (150%, or 300ppm)



ASSAY OF MARKETED FORMULATION

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using afore mentioned formula.

Fig. No. 16 Chromatogram of Sample -1

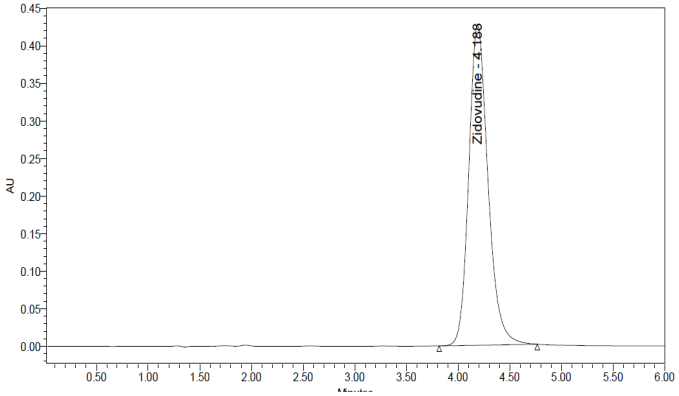
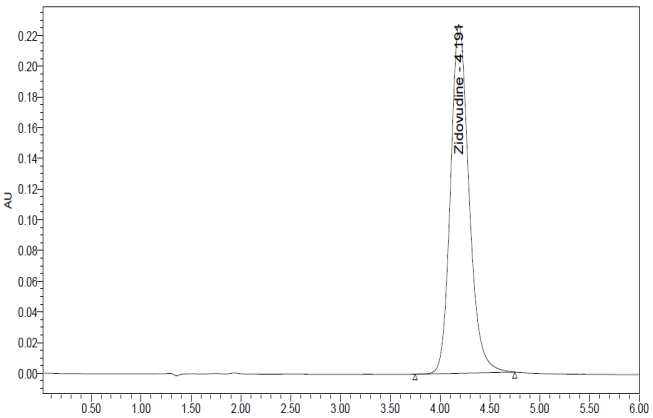


Fig. No. 17 Chromatogram of Sample -2



RESULTS AND DISCUSSIONS

Zidovudine is the drug mainly used to treat AIDS. A simple reverse phase HPLC method was developed for the determination of Zidovudine. Column Kromocil (150 x 4.6 mm, packed with 5µm) with mobile phase Buffer: Methanol (60:40) was used. The flow rate was 1.3ml/ min and effluent was monitored at 266 nm. The column temperature was 25°C. The Retention time was found to be3.396.

System Suitability Parameters

Table No

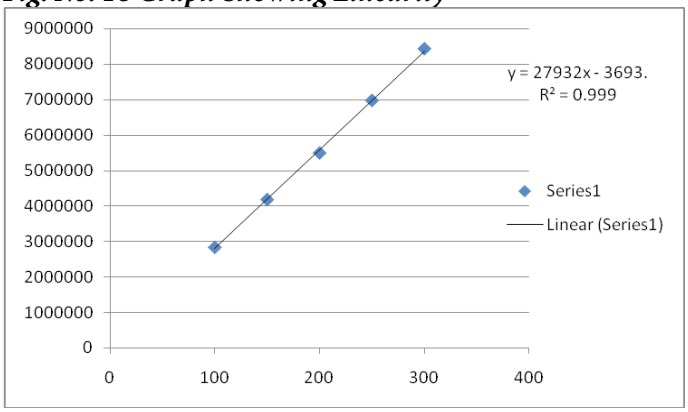
	Retention Times	Peak Area	Tailing Factor	Theoretical Plates
1	3.378	5554712	1.13	2693
2	3.385	5506629	1.14	2709
3	3.394	5514157	1.14	2671
4	3.408	5505305	1.14	2685
5	3.788	5514960	1.15	2060
Mean	3.3906	5519152	1.14	25636
SD	0.011304	20344.54	-	-
%RSD	0.333416	0.3686	-	-

Linearity

Table No

Linearity Level (%)	Concentration (ppm)	Area
50	100	283837\8
75	150	4182382
100	200	5494170
125	250	6972430
150	300	8426416

Fig. No. 18 Graph Showing Linearity



Optimized characteristics for linearity of Zidovudine by RP-HPLC

Parameters	Observed values
Linearity concentration	100 – 300 ppm
Slope	27932
Intercept	-3693
Correlation coefficient	0.999

Assay of Zidovudine

Table No

Sample No	*Sample Areas*	*%Assay*	*%Avg.Assay*
1.	5508513	99.63%	99.8%
2.	5533289	100.07%	

From the Chromatograms it was found that the sample and standard retention times are similar i.e3.378 to 3.788. From linearity Table 1, it was found that the drug obeys linearity within the concentration range of 100-300ppm for zidovudine By using this method assay of marketed formulated was carried out and it is found to be as 99.8%.

SUMMARY AND CONCLUSION

Zidovudine is the drug used in the treatment of AIDS. It is a nucleoside & nucleotide reverse transcriptase inhibitor.

From literature review and solubility analysis initial chromatographic conditions Mobile phase acetate buffer: methanol 20:80 were set (Buffer PH 5.3 adjusted with glacial acetic acid), Kromocil 150*4.6mm Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 266 nm. As the methanol content was increased Zidovudine got eluted with good peak symmetric properties. Mobile phase buffer: Methanol (60:40), Column Kromocil 150*4.6mm 5µm and flow rate 1.3ml/min, detection wave length 266nm, column temperature 25oC and diluent water: methanol (90:10) conditions were finalized as optimized method.

System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria.

Linearity study was carried out between 50% to150 % levels, R2 value was found to be as 0.999.

By using above method assay of marketed formulation was carried out,99.8% was present.

Full length method was not performed; if it is done this method can be used for routine analysis of Zidovudine

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