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ISSN: 0975-7538
Research Article

Stability indicating method for simultaneous determination of polar and non-polar related compounds of Zidovudine in drug substance and drug product tablet form

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ABSTRACT

A Simple, rapid and sensitive method has been developed and validated for the determination of polar and non-polar related compounds of Zidovudine (ZVD) in drug substance and drug product. Efficient chromatographic separation was achieved on a Symmetry C18 column with a simple mobile phase combination containing a gradient mixture of solvent A and B at a flow rate of 1.0 mL min^{-1} and quantitation was carried out using ultraviolet detection at 259 nm with ambient column temperature. The resolution between ZVD and its related compound-B was found to be greater than 2.2. Regression analysis shows an "r" value (correlation coefficient) greater than 0.999. This method was capable to detect all the process impurities of Zidovudine, at a level below 0.05% with respect to a test concentration of 0.50 mg mL^{-1}. The inter-day and intra-day precisions for all impurities of ZVD were found < 2.0% of Relative standard deviation (RSD). The method has consistent recoveries. The ZVD and its tablet form were subjected to stress conditions of acid, base, oxidative, water hydrolysis, photolysis, thermal, and humidity degradation, as prescribed by International conference on harmonization (ICH).

Keywords: Stability indicating; Zidovudine; Polar and Non-polar related Compounds; Drug Substance; Drug product - Tablet formulation.

INTRODUCTION

Zidovudine (ZVD) formerly called azidothymidine, a pyrimidine nucleoside analogue active against HIV-1, 1-(3-azido-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione. ZVD is a white to beige, odorless, crystalline solid. The molecular formula is $C_{10}H_{13}N_5O_4$. ZVD is a nucleoside reverse transcriptase inhibitor, is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection. Zidovudine is a synthetic nucleoside analogue. Intracellular, zidovudine is phosphorylated to its active 5'-triphosphate metabolite, zidovudine triphosphate (ZDV-TP). The principal mode of action of ZDV-TP is inhibition of reverse transcriptase (RT) via DNA chain termination after incorporation of the nucleotide analogue. ZDV-TP is a weak inhibitor of the cellular DNA polymerases 'a' and 'y' has been reported to be incorporated into the DNA of cells in culture.

In the literature, the determination of ZVD by HPLC during stability studies has been published with only one known impurity - thymine is established (Saranjit.S et al, 2005) and some methods are published in UV (Sibel A.O et al, 2002; Sudhakar Reddy et al, 2012). There are few other methods are published with combination of different type of drugs by HPLC (Vander H.Y et al, 2011; Adams. E et al, 2009; John A.D et al, 2000; Venkata Reddiah CH, 2012) Most of the publications are available from bio analytical studies (Soumya.S et al, 2006; James T.S et al, 2002; Remon J.P et al, 2009; Zhen Wu et al, 2008; Michael G.B et al, 2007; Sekar R et al, 2005; Luisa V et al, 2002; Parthiban. C et al, 2012; Tarinas A et al, 2007) As per United States Pharmacopeia (USP) and European Pharmacopeia (Ph. Eur. / EP) monographs (United States pharmacopeia, 35th edn) (European pharmacopeia, 6th edn), the determination of related compounds of ZVD is mentioned in two methods (polar impurities by HPLC and non-polar impurities by TLC). There were no LC methods have been reported on the determination of two methods in a single method. Hence, developed a stability-indicating LC method that can separate and determine the Thymine (USP, EP Related Compound – C / Polar Impurity / Imp-1), Thymidine (Unspecified Impurity / Polar Impurity / Imp-2), Related Compound – A (USP, EP Polar Impurity / Imp-3), Related Compound – B (USP, EP Polar Impurity / Imp-4), Triphenylmethanol (TPM) (EP Related Compound D / Non Polar Impurity / Imp-5).

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Received on: 10-03-2015
Revised on: 19-03-2015
Accepted on: 23-03-2015

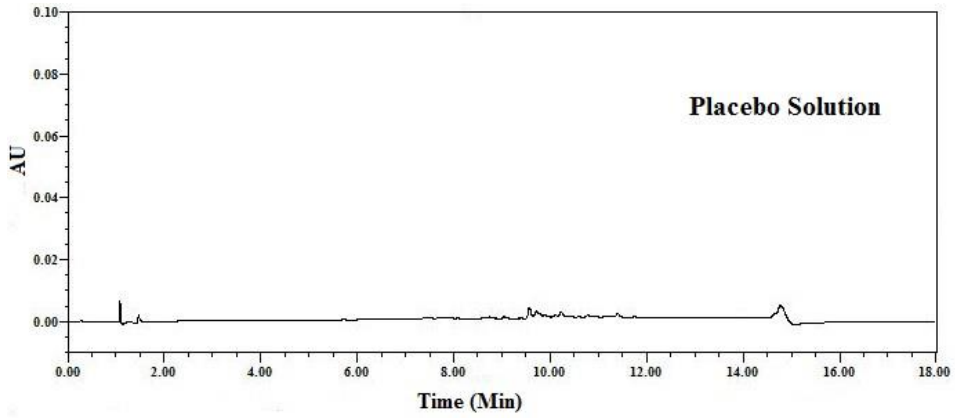


Figure 1: Typical Chromatogram of Placebo Solution

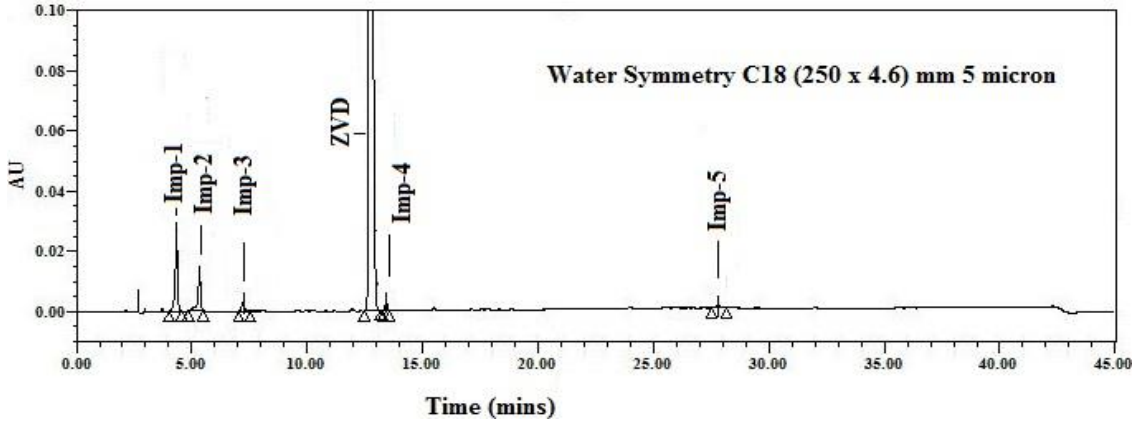


Figure 2a: Typical gradient program with methanol and water

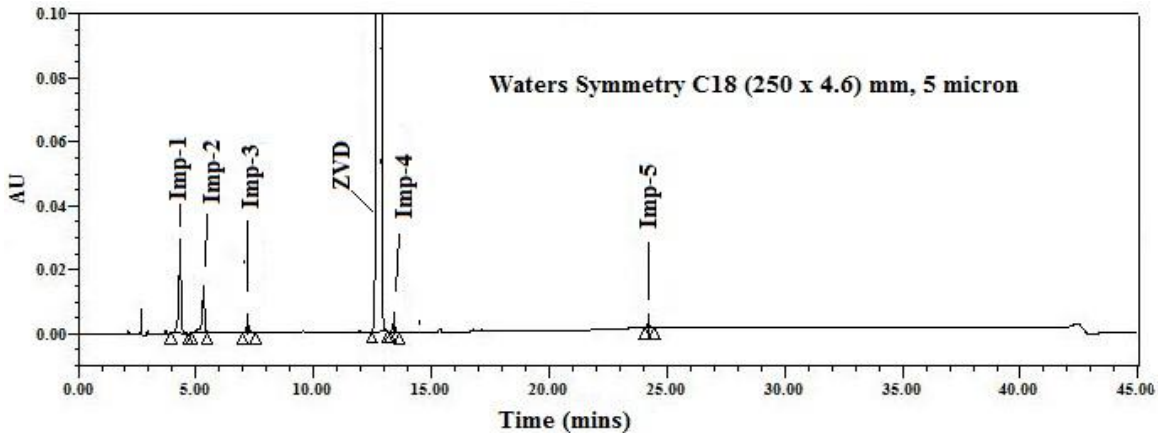


Figure 2b: Typical gradient program with acetonitrile and water

The Chemical names and structures of ZVD / Related compounds were mentioned in Table.1.

EXPERIMENTAL

1. Chemicals and reagents

The purity of ZVD Standard is about 99.9% and its impurities viz. Imp-1 (99.9%), Imp-2 (99.8%), Imp-3 (100.0%), Imp-4 (100.0%), Imp-5 (99.9 %). HPLC-grade Methanol, Acetonitrile procured from Honeywell. Ammonium Acetate and 1-Octane sulfonic acid sodium salt chemicals of analytical reagent grade were procured from Spectrum. Waters Symmetry C-18 column was procured from Waters Associates Inc.

2. Equipment

Chromatography was performed with Waters Alliance HPLC system (Milford, USA) consists of quaternary pump equipped with a 2695 separation module with inbuilt auto injector and 2996 photodiode array detector. The output signal was monitored and processed using empower3 software. Cole Palmer digital water bath was used for hydrolysis studies. All solutions were degassed using ultra-sonication (Branson) and filtered through a 0.45µm membrane filter (PALL Life sciences, USA). Intermediate precision was performed on different make of HPLC system (SHIMADZU, Kyoto, JAPAN) equipped with single wavelength UV Detector.

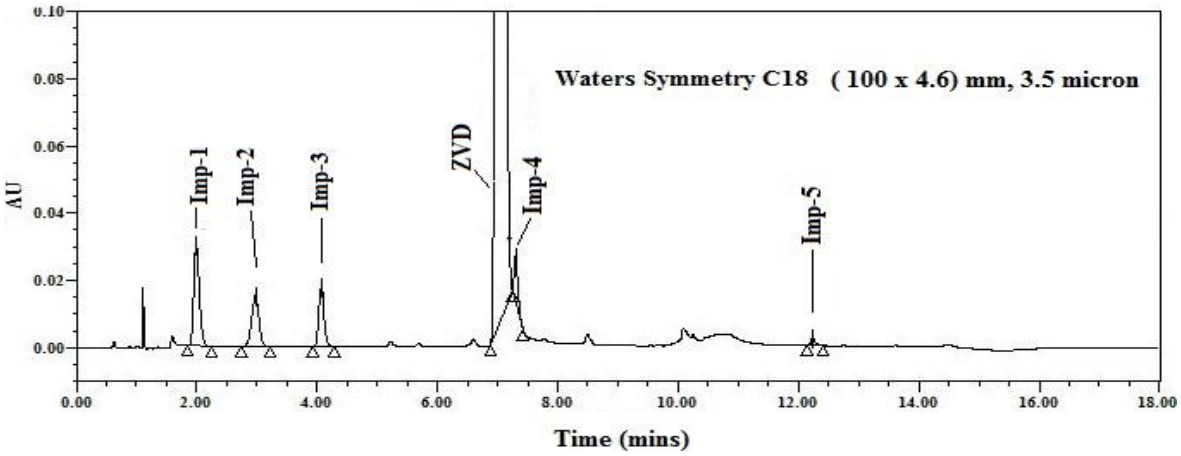


Figure2c: Typical gradient program with ammonium acetate buffer and acetonitrile

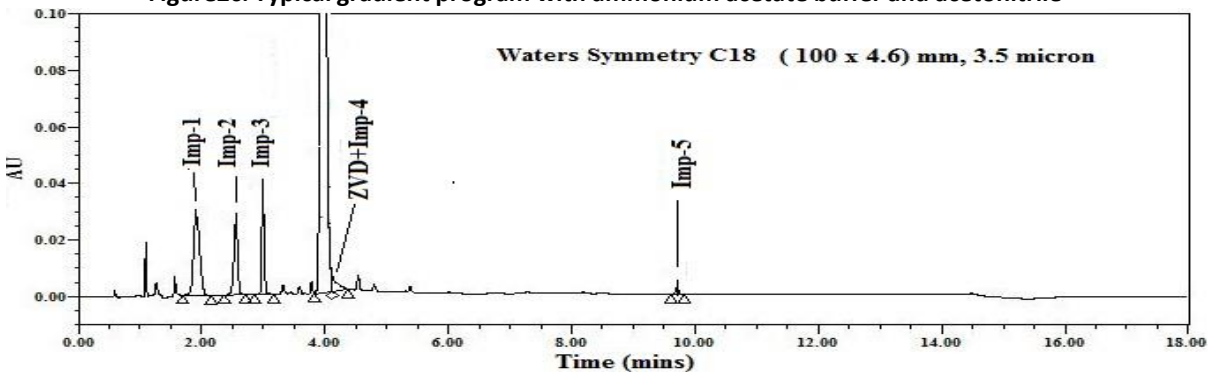


Figure 2d: Typical gradient program with 1-octane sulfonate sodium salt buffer, acetonitrile and methanol

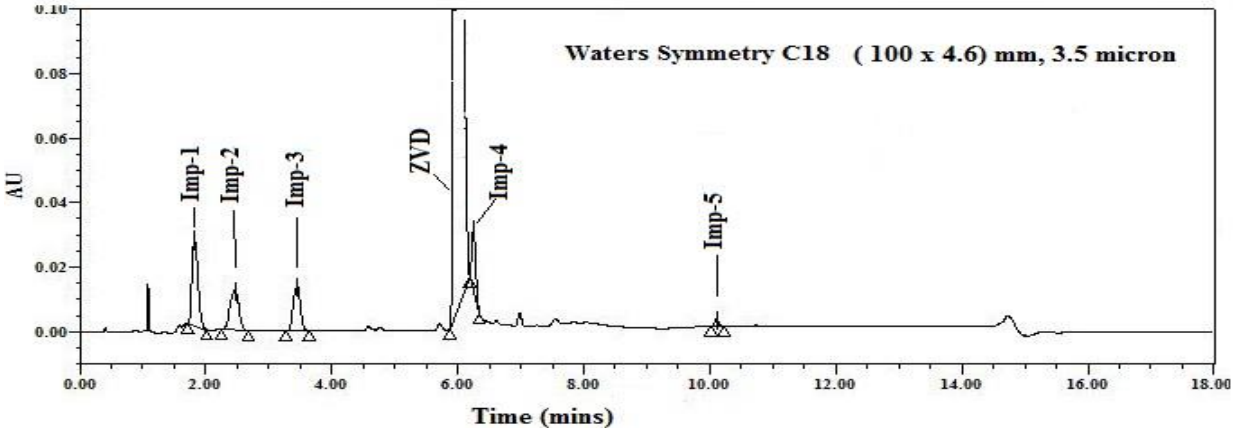


Figure 2e: Typical gradient program with 1-Octane sulfonate sodium salt buffer, acetonitrile and methanol

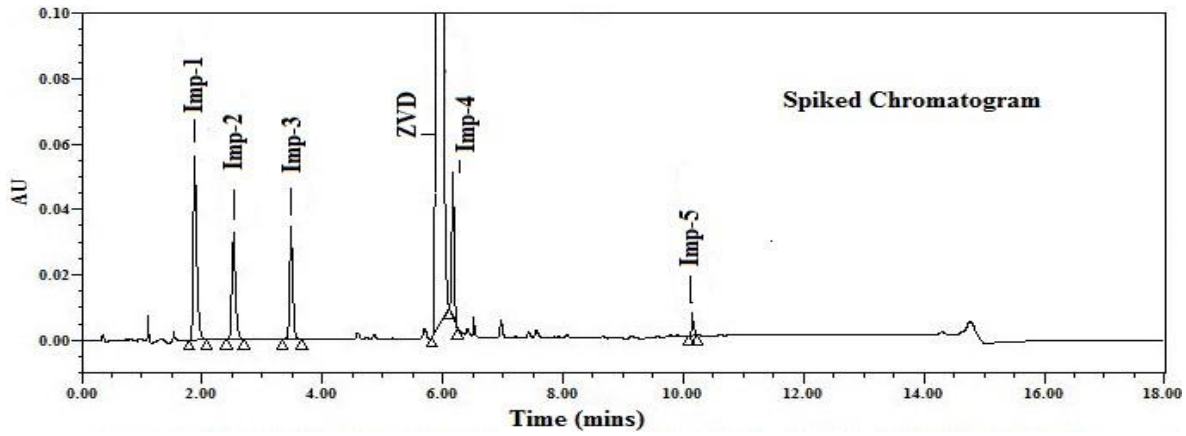


Figure 3: Typical Chromatogram of Drug product sample spiked solution

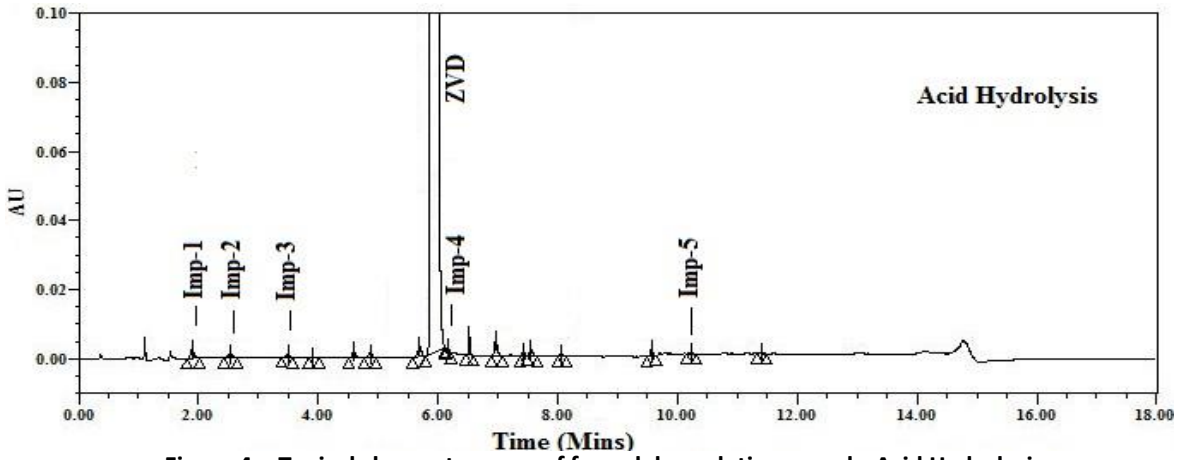


Figure 4a: Typical chromatograms of forced degradation sample-Acid Hydrolysis

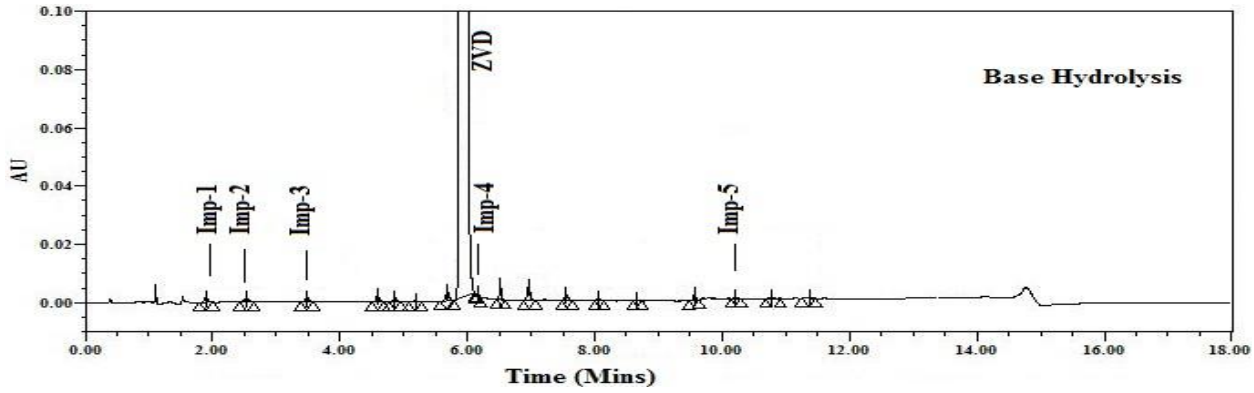


Figure 4b: Typical chromatograms of forced degradation sample-Base Hydrolysis

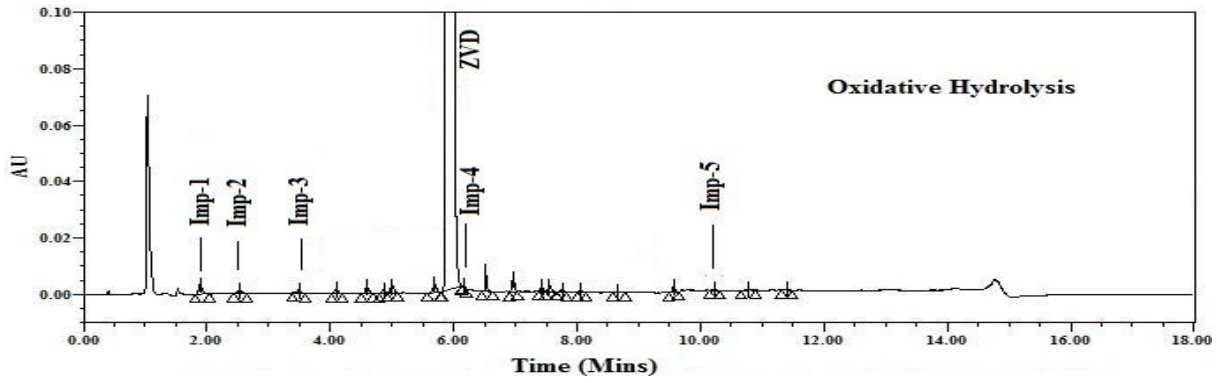


Figure 4c: Typical chromatograms of forced degradation sample -Oxidative Hydrolysis

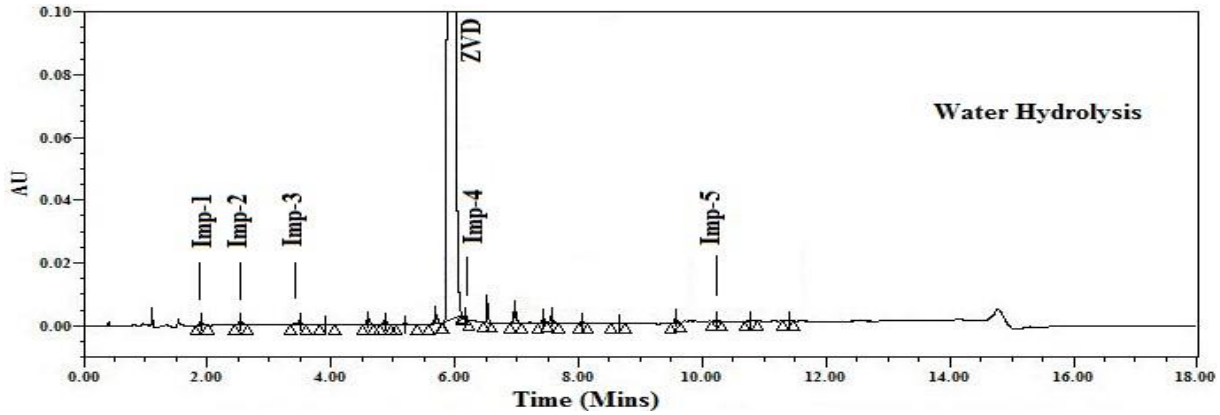


Figure 4d: Typical chromatograms of forced degradation sample-Water Hydrolysis

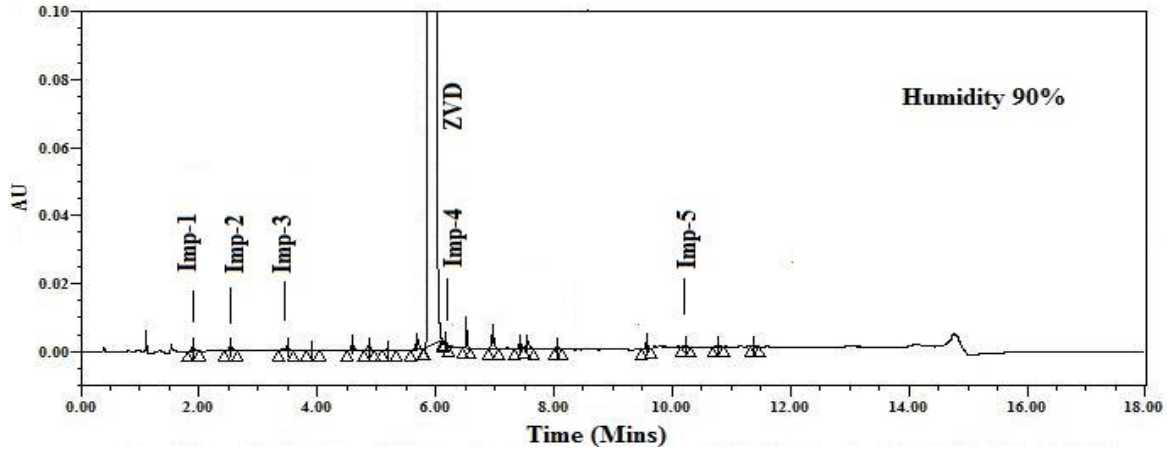


Figure 4e: Typical chromatograms of forced degradation sample-Humidity Stress

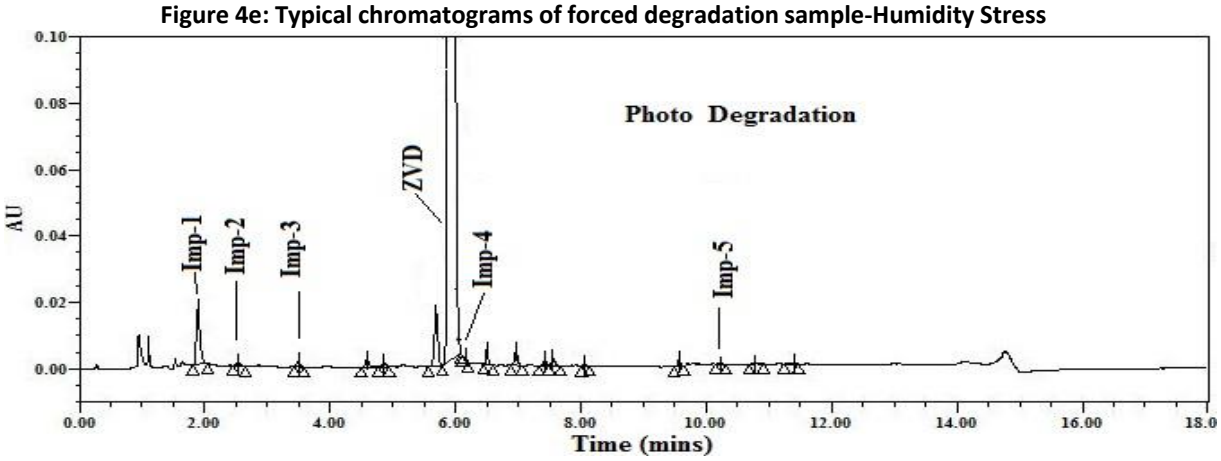


Figure 4f: Typical chromatograms of forced degradation sample-UV light Stress

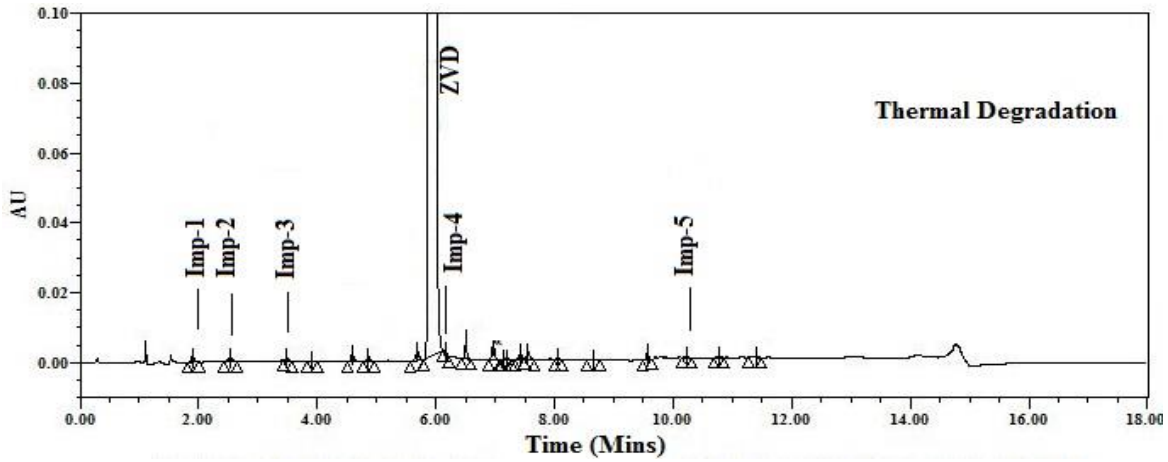


Figure 4g: Typical chromatograms of forced degradation sample-Thermal Stress

3. Chromatographic conditions

The method was developed on using Waters Symmetry C-18, 100mm length, i.d-4.6mm, 3.5µm particle size column with mobile phase containing a gradient mixture of solvents A and B. Buffer consists of 0.02M ammonium acetate with 0.2% of 1-Octane sulfonic acid sodium salt, Solvent A consists of Buffer : Methanol :

Acetonitrile in the ratio of 910 : 50 : 40 (*v/v/v*) and B in the ratio of 100 : 800 : 100 (*v/v/v*) respectively. The LC gradient program (T/%B) was set as 0.01/0, 4/25, 8/100, 13/100, 14/0, 18/0. The flow rate of the mobile phase was 1.0 mL min^{-1} and the wavelength was monitored at 259 nm. The injection volume was 25 µL. The diluents used as Water and Methanol in the ratio of (800 : 200) (*v/v*).

4. Preparation of standard solution

Stock solutions of ZVD (0.1 mg mL^{-1}) were prepared by dissolving appropriate amount of drug in diluents. Diluted standard was prepared 0.0025 mg mL^{-1} with respect to ZVD test concentration.

Table 1: Chemical names and structures for ZVD and its related Compounds

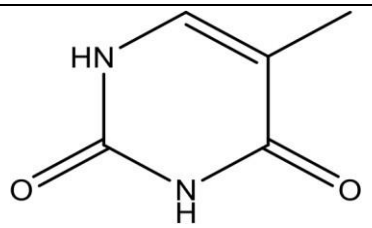
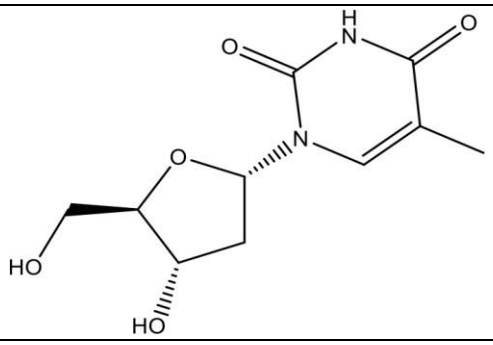
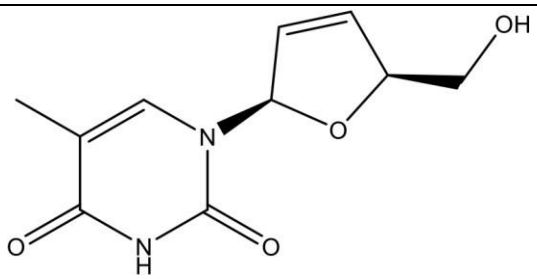
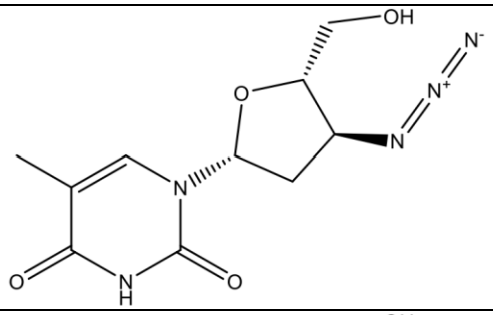
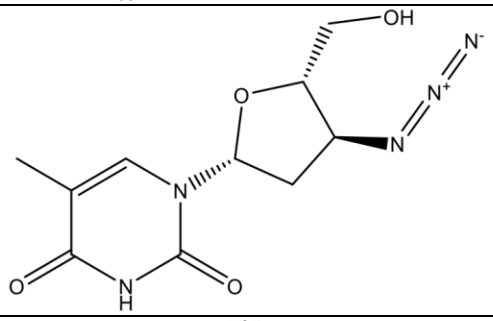
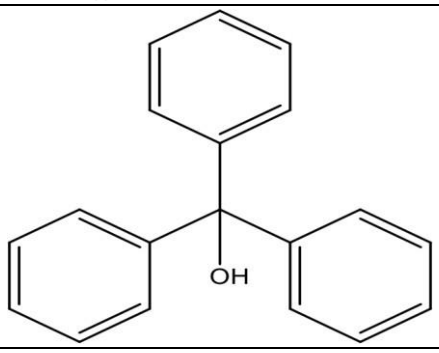
Name of the Compound	Structure
A) 5-methylpyrimidine-2,4(1H,3H)-dione. (Thymine) (USP / EP Related Compound C) (Impurity – 1)	
B) 1.1-(2-Deoxy-b-D-erythro-pentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione. (Thymidine) (Unspecified impurity / Degradent)(Impurity – 2)	
C) 1-[(2R,5S)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione. (USP / EP Related Compound A)(Impurity – 3)	
D)1-(3-azido-2,3-dideoxy-β-D-erythro-pentofuranosyl)-5-methylpyrimidine- 2,4(1H,3H)-dione. (Zidovudine / ZVD)	
E) 1-(3-chloro-2,3-dideoxy-β-D-erythro-pentofuranosyl)-5-methylpyrimidine-2,4(1H,3H)-dione. (USP / EP Related Compound B) (Impurity – 4)	
F)Triphenylmethanol (USP Related Compound / EP Related Compound D) (Impurity – 5)	

Table 2: Chromatographic evaluation data

S No	Compound	RRT [a]	RRF [b]
1	Impurity-1	0.32	2.55
2	Impurity-2	0.43	1.48
3	Impurity-3	0.59	1.46
4	ZVD	1.00	1.00
5	Impurity-4	1.04	1.54
6	Impurity-5	1.71	0.25

[a] Relative retention times (RRT) were calculated with respect to (w.r.t) ZVD peak.
[b] Relative response factor were calculated w.r.t ZVD peak.

Table 3: Evaluation of Forced Degradation

Name of related compound	RRT	% Degradation in stress condition							
		Sample As is	Acid	Base	Oxidative	Water	Thermal	Humidity	Photolytic
Imp-1	0.32	0.03	0.1	0.1	0.13	0.16	0.14	0.03	0.41
Imp-2	0.43	0.03	0.1	0.12	0.11	0.09	0.03	0.03	0.03
Imp-3	0.59	0.04	0.02	0.02	0.03	0.03	0.03	0.03	0.04
unknown	0.66	ND	ND	ND	0.1	ND	ND	ND	ND
unknown	0.78	0.04	0.1	0.04	0.13	0.04	0.12	0.1	0.11
unknown	0.96	0.07	0.11	0.07	0.07	0.07	0.07	0.07	0.36
Imp-4	1.04	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Imp-5	1.72	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
* ND - Not Detected									

Table 4: Peak purity summary

Stress Condition	ZVD			
	% Mass Balance	Purity Angle	Purity Threshold	Purity Flag
Acid Hydrolysis	98.2	7.844	9.655	No
Base Hydrolysis	99.1	7.844	9.634	No
Hydrolysis	98.9	7.883	9.770	No
Oxidative Hydrolysis	99.2	7.802	9.589	No
Thermal	98.1	7.761	9.594	No
Humidity	98.5	7.626	9.596	No
Photolytic UV	99.0	7.802	9.589	No

5. Sample preparation for Drug substance / Drug product

ZVD or ZVD tablets, crushed tablet powder equivalent to 100 mg of ZVD was accurately weighed and transferred into 200 mL volumetric flask. Added 150 mL of diluents and sonicated for 20 min with occasional swirling and diluted. An aliquot of sample centrifuged for 10 min. Sample solution contains ZVD Concentration is about 0.5 mg mL^{-1} concentration.

6. Placebo preparation for tablet formulation

An accurately weighed quantity of placebo equivalent to the amount present in the tablet sample preparation (equivalent to 100mg of ZVD) into 200 mL volumetric flask. Added 150 mL of diluents and sonicated for 20 min with occasional swirling and diluted. An aliquot of sample centrifuged for 10 min. typical chromatogram of placebo solution was presented in Fig.1.

7. Relative Response Factor Determination

Five different concentrated solutions of all the five known impurities and ZVD were prepared in the diluents and studied the each slope of the respective compound with respect to response vs concentration as per chromatography conditions. The relative response factor (RRF) for each individual impurity was calculated using the ratio of slope of respective known impurity to the active drug substance. Chromatographic evaluation data was presented in Table.2.

RESULTS

1. Method development

From the pharmacopeias of USP, EP, it was observed that two methods for the determination of impurities as Imp-1, Imp-3, Imp-4 and Imp-5 (polar and non-polar related compounds) by HPLC and by TLC respectively. There are another publication which defines the 'stability studies by HPLC method for determination of degradation product viz. only Imp-1 in ZVD drug substance'.

From these two aspects taking into consideration there is an evidence to develop the single method to determine all the possible degradation products viz. polar and non-polar related compounds of ZVD drug substance and drug product. Hence, the method develop-

ment experiments have been originated from the USP method.

Initially columns were chosen as waters symmetry C18 and inertsil C8-3 (4.6 X 250) mm 5µm. All impurities and ZVD wavelengths maxima observed at 259 nm. Mobile phase chosen as water / methanol and water / acetonitrile in isocratic elution with different compositions, observed that the Imp-5 peak is not eluted up to 50 min runtime and poor resolution was observed between ZVD and Imp-4. Gradient method was opted for the elution of all impurities with appropriate retention time and resolution. In gradient, mobile phase as water, methanol and acetonitrile with different proportions and as well C18 with different length columns were chosen for different experiments. As above gradient methods, fronting (Imp-1 & Imp-2) peak shape and poor resolution (ZVD & Imp-4) was observed Fig.2.

The optimization of organic phase was studied based on the elution pattern of the components along with symmetric peak shapes (Krishnaiah.Ch, et al, 2010, 2011). More proportion of methanol was chosen rather than the acetonitrile due to its compatibility with drug molecule and good resolution between peaks.

In order to improve peak shapes and resolutions, introduced different buffer strengths (different pH & salts) instead of water and the same experiments were conducted. In the above experiments, the buffers like Sodium, potassium buffers yields not satisfactory peak shapes and resolution. Ammonium buffer and other composition of solvents resulted good peak shapes but poor resolution was observed. To maintain appropriate resolution between ZVD and Imp-4, introduced an ion pair reagent. In that proportion, the ion pairs can enhance selectivity because of hydrogen bonding capacity and also offer electrostatic interaction with analytes of opposite charges. With the addition of the ion pair reagent into the mobile phase and the use of an octadecyl silane column, the resolution of ZVD and Imp-4 increased as expected.

The Final experiment has been carried with ratios of Solvent A and Solvent B. Buffer consists of 0.02M ammonium acetate with 0.2% of 1-Octane sulfonic acid sodium salt, Solvent A consists of Buffer : Methanol : Acetonitrile in the ratio of 910 : 50 : 40 (*v/v/v*) and B in the ratio of 100 : 800 : 100 (*v/v/v*) respectively. The LC gradient program (T / %B) was set as 0.01/0, 4/25, 8/100, 13/100, 14/0, 18/0 and maintained flow as 1.0 mL min^{-1}. Loaded the spiked solution into system, it was observed that good peak shapes and resolution (ZVD and Imp-4) with appropriate (short) retention times. Retention times as follows: Imp-1, Imp-2, Imp-3, ZVD, Imp-4 and Imp-5, 1.88 min, 2.52 min, 3.48 min, 5.94 min, 6.17 min, 10.16 min respectively. Typical spiked chromatogram was presented in Fig.3.

2. Validation of the Method

The proposed method was validated as per ICH guidelines (ICH, 2005).

2.1. Precision and intermediate precision

Precision of the method is verified by repeatability. Repeatability was checked by injecting six individual preparations of ZVD and its tablet blend sample spiked at 0.5% of all its known impurities with respect to its active drug concentration present in sample. The intermediate precision of the method was also evaluated using different analyst and different instrument and by performing the six individual preparations by spiking the impurities on ZVD and its tablet blend sample. % RSD for each impurity concentration was calculated and observed less than 5.0 % for all its known impurities in precision & intermediate precision studies.

2.2 Specificity

Specificity is the ability to assess clearly the analyte in the presence of components that may be expected to be present such as impurities, degradation products and matrix components.

2.2.1. Placebo Interference

Chromatogram of placebo preparation and sample preparation was cross checked and found that there was no peak interference at the retention times of ZVD and its known impurities.

2.2.2. Forced degradation

The specificity studies were performed at an initial concentration 0.5 mg mL^{-1} of ZVD as well as individual drug substances to provide an indication of the stability-indicating property and specificity of the proposed method. PDA detector was used to make sure the homogeneity and purity of the active drug along with known impurity peaks. Intentional degradation was attempted to stress condition of acid (0.1N HCl at 60°C / 30min), base (0.1N NaOH at 60°C / 30min), hydrolytic (60 °C / 1Hr), oxidation (1.0 % H_2O_2 at 40°C / 1Hr), thermal (100°C / 24Hrs), humidity (90% RH, 15 Days) and photolytic (UV / Not less than 200 watt hours-square $meter^{-1}$) to evaluate the ability of the proposed method to separate the impurities of ZVD from its potential degradents. Peak purity test was carried out for the ZVD peaks as well as known impurities by using PDA detector in stress samples. Degradation data was found in almost all above stress conditions and presented in Table.3. The assay of stressed samples was calculated against a qualified reference standard and the mass balance was found close to 98% (% assay + % sum of all its related compounds + % sum of all its degradants respectively). Typical specificity chromatograms and its mass balance data were presented in Fig.4 & Table.4 respectively.

2.3. Accuracy

Standard addition and recovery experiments were conducted on real sample to determine accuracy of the

related compounds method. The study was carried out in triplicate sample preparation using five concentration levels from LOQ - 0.25 µg mL^{-1} (0.05%) to 5 µg mL^{-1} (200%) for ZVD related compounds. The percentage recovery of impurities in ZVD and its tablet blend samples varied from 93.5 % to 107.2%.

2.4. Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ for ZVD and its impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. LOD was obtained at 0.025% level where as LOQ was obtained at 0.05% level with respect to its active test concentration (0.5 mg mL^{-1}). Precision and accuracy study was also carried out at the LOQ level by preparing the six individual spiked preparations and the results are found to be well within the limits.

2.5. Linearity

Linear calibration plot for the related compounds method was established at 5 determinations over the calibration range tested, i.e. LOQ (0.05%) to 1.0% for impurities with respect to its active drug concentration (0.5 mg mL^{-1}). Linearity test solutions were prepared by spiking the pure stock solutions of the drug substance and its related compounds at five concentration levels from 10% (LOQ) to 200% of the impurity specification level (LOQ-0.05%, 0.1, 0.2, 0.5 and 1.0%) viz. targeted % impurity level as 0.5%. The peak response vs. concentration data was treated by least-squares linear regression analysis. Calibration graphs were plotted for ZVD and its related compounds and observed that the correlation coefficient found greater than 0.999. The result shows that an excellent correlation existed between the peak area vs. concentration of ZVD and its impurities respectively.

2.6. Solution stability & Mobile phase stability

The stability of ZVD related compounds in solution for the related compounds method was determined by leaving precision spiked sample solution and diluted standard solution at room temperature for 48 h and measuring the amounts of the five impurities at every 48 h. The stability of mobile phase was also determined by analyzing freshly prepared solution of ZVD impurities at 24 h intervals for 48 h. The sample diluted standard solution and mobile phase are stable up to 48 h at room temperature.

2.7. Robustness

In all the deliberative varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), the analyte and impurity peaks were well resolved and elution orders were remained unchanged. The USP resolution between ZVD and Impurity-4 was found more than 2.2. Tailing factor for ZVD & its impurities was less than 2.0. The variability in the estimation of ZVD & its impurities was well within ±5%.

DISCUSSION

A simple and new analytical short RP-LC method has been developed for ZVD and its associated related compounds for drug substance and its solid dosage forms. This method can be able to separate all the process and degradation impurities. Satisfactory results were observed from the validation data. The described method can be able to quantify all the impurities up to 0.05% level. The cycle time (18 min) enables rapid determination of the drug. In robust 5% composition variation of organic solvents in the mobile phase, there was no impact in resolution and quality in quantifying the impurities. This method has been validated as per ICH and proved specific. This method could be useful for reaction monitoring for the synthesis of ZVD and its dosage forms. This testing method can also be employed in real time (25°C/60%RH), intermediate (30°C/65%RH) and accelerated stability conditions (40°C/75%RH).

CONCLUSION

A simple, rugged and robust gradient RP-HPLC method was developed for separation and quantitative determination of ZVD, its associated polar and non-polar impurities. The method is precise, accurate and selective. The method was completely validated and showed satisfactory data from all the validation parameters. The developed method is stability indicating and can be used to assess in-process and final drug product testing.

ACKNOWLEDGEMENTS

The authors are thankful to the Research and Development team for providing laboratory facility for this research work.

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