Trade Science Inc.

*September 2009* *Volume 8 Issue 3*

Analytical CHEMISTRY

An Indian Journal

Full Paper

**ACAIJ, 8(3) 2009 [360-367]**

# Determination of zaleplon in the presence of its degradation products by a stability indicating UPLC method

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*Received: 3rd July, 2009 ; Accepted: 13th July, 2009*

## ABSTRACT

A new, simple, sensitive, stability indicating isocratic RP-UPLC assay method has been developed for the quantitative determination of Zaleplon in the presence of its degradation products . Efficient Chromatographic separation was achieved on a C18 stationary phase with simple mobile phase Combination of water and Methanol and quantitation was carried out using ultraviolet detection at 230 nm with a flow rate of 0.4 mL $min^{-1}$. In the developed UPLC method the resolution ($R_s$) between Zaleplon and its all degradants obtained from forced degradation studies were found to be greater than 2.0. Regression analysis shows an $r^2$ value (correlation coefficient) of greater than 0.99 for Zaleplon And it's all the degradant impurities. This method was capable to detect the Zaleplon in presence of the Degraded impurities The inter and intra day precision values Zaleplon was found to be within 2.0 % RSD. The method has shown good and consistent recoveries for Zaleplon in bulk drugs (99.3-101.4 %). The test solution was found to be stable in diluent for 48 h. The drug substances were subjected to Stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable Degradation was found to occur in acid stress, base stress and oxidative conditions. The stressed test Solutions were assayed against the qualified working standard of Zaleplon and the mass balance in Each case was close to 99.8% indicating that the developed method was stability-indicating. The Method was developed and optimized by analyzing the forcefully degraded samples The developed RP-UPLC method was validated with respect to linearity, accuracy, precision and ruggedness.



## KEYWORDS

Column liquid chromatography;
Zaleplon;
Forced degradation;
Validation;
Stability indicating.

## INTRODUCTION

Zaleplon (*N*-(3-(3-cyanopyrazolo [1,5-*a*] pyrimidin-7-yl)phenyl)-*N*- ethylacetamide, (Figure 1) is a sedative/hypnotic, mainly used for . It is a nonbenzodiazepine hypnotic from the pyrazolopyrimidine class. Zaleplon is one of few sleep medications which have been found to not cause an increase in road traffic accidents, thus demonstrating a much higher safety profile than many other hypnotics currently on the market. Zaleplon has a pharmacological profile similar to benzodiazepines that is characterized by an increase in SWDS with rapid onset of hypnotic action. Zaleplon is a full agonist for the benzodiazepine α1 receptor located on the $GABA_A$ receptor ionophore complex in the brain, with lower affinity for the α2 and α3 subtypes. It selectively enhances the action of GABA similar to but more selectively than benzodiazepines. Zaleplon, although not benzodiazepine-like in chemical structure induces sedative-hypnotic, anticonvulsant and anticonflict effects via its binding to the central nervous system (CNS) type benzodiazepine receptors. The elimination half life of zaleplon is 1 hour. Absorption is rapid. Zaleplon can be classed as an ultra short acting sedative hypnotic drug for the treatment of insomnia characterized by difficulty in falling asleep. Zaleplon increases EEG power density in the delta frequency band and a decrease in the energy of the theta frequency band. In tests on rabbits zaleplon shows drowsy pattern of spontaneous EEG characterized by high-voltage slow waves and desynchronization of hippocampal theta waves and an increase in the energy of the delta frequency band on the spectral analysis of the electroencephalogram

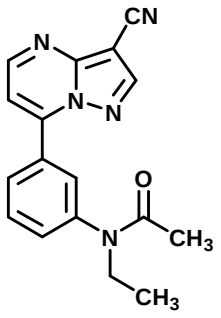


**Figure 1 : Chemical structures and labels of Zaleplon**

Extensive literature survey did not reveal any simple, sensitive and stability indicating LC method for quantitative estimation of Zaleplon in bulk drugs. Few LC-MS methods were reported in literature describing the determination of Zaleplon in biological fluids[1-6], However there is no stability indicating methods for the quantitative assay of Zaleplon in bulk drugs. An ideal stability indicating chromatographic method should estimate the drug and is able to resolve from its potential impurities and degradation products. The present drug stability test guideline Q1A (R2)[7] issued by International Conference on Harmonization (ICH) suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to separation of degradation products and hence supporting the suitability of the proposed analytical procedures According to ICH, stress testing of the drug substance can help the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used. The nature of stress testing will depend o the individual drug substance and it is likely to be carried out on a single batch of the drug substance. It also requires that analytical test procedures for stability samples should be stability indicating and they should be fully validated as per ICH Q2 (R1)[8] and USP/NF[9].

Hence, an attempt has been made to develop an accurate, rapid, specific and reproducible method for the determination of Zaleplon in the presence of its degradation products, along with method validation as per ICH norms.

## EXPERIMENTAL

### Chemicals

Samples of Zaleplon and were procured USP-India, Hyderabad, India (Figure 1). HPLC grade Methanol Analytical reagent grad, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system. All samples used in this study were of greater than 99.0% purity.

### Equipment

The LC system used for method development, forced degradation studies and method validation was Waters ACQUITY UPLC Binary solvent Manager plus auto sampler and a photo diode array detector. The

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output signal was monitored and processed using Empower software on Pentium computer (Digital equipment Co). Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Mack Pharmatech, Hyderabad, India).

### Chromatographic Conditions

The chromatographic separation was performed on an Acquity UPLC® BEH C18 Column (100 x 2.1) mm with 1.7 µm particles. The mobile phase comprising a mixture of water & methanol in the ratio (65:35 v/v), at a flow rate of 0.4 mL $min^{-1}$.with isocratic elution. The column temperature was maintained at 25°C and the detection was monitored at a wavelength of 230 nm. The injection volume was 2 µL. Pure Methanol was used as diluent.

### Preparation of Solutions

### Preparation of Standard Solutions

A stock solution of Zaleplon (5.0 mg $mL^{-1}$) was prepared by dissolving appropriate amount in the diluent. Working solutions of 100 µg $mL^{-1}$ were prepared from above stock solution for assay determination.

### Preparation of Assay Solutions

Transferred about 50-mg of Zaleplon sample into 50 -mL volumetric flask, dissolved in, and diluted to volume with diluent.Transferred 5-ml of this solution to a 50-ml volumetric flask mixed & make up to the volume with the diluent to obtain a solution of 100 µg $mL^{-1}$

### Analytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, ruggedness, and system suitability.

### Specificity/ Application of Stress (Forced Degradation Study)

Specificity of the developed method was assessed by performing forced degradation studies.. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

The specificity of the developed UPLC method for Zaleplon was determined in the presence of its degradant impurities. Forced degradation studies were performed on Zaleplon to provide an indication of stability indicating property and specificity of the proposed method[10,11]. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), heat (100°C), acid hydrolysis (1N HCl), base hydrolysis (0.1N NaOH), water hydrolysis and oxidation (3 % $H_2O_2$). For heat and light studies, study period was 10 days where as for acid it was 8 hrs at 80°C, and oxidation it was at 80°C for 3 h and for base it was at 80°C for 2 h. Peak purity of stressed samples of Zaleplon was checked by using a 2996 photo diode array detector (PDA) from Waters. The purity angle within the purity threshold limit demonstrates the analyte peak homogeneity.

Assay studies were carried out for stressed samples against the qualified reference standard and the mass balance (% assay + % of degradation products + % of impurities) was calculated. Assay analysis was also performed on some batch samples.

### Analytical Method Validation

### Precision

Assay method precision was evaluated by carrying out six independent assays of test sample of Zaleplon against qualified reference standard. The % RSD of six assay values obtained was calculated. The intermediate precision (ruggedness) of the assay method was evaluated by different analyst and by using different column on different days from the same laboratory.

### Linearity

Linearity test solutions for assay method were prepared from stock solution at five concentration levels from 25 to 200 % of assay analyte concentration (25, 50, 75, 100, 150 and 200 µg $mL^{-1}$). The peak area versus concentration data was collected and performed regression analysis by the method of least squares. The Correlation coefficient, Slope & y-intercept values were calculated from the calibration plot obtained.

### Accuracy

The accuracy of the assay method was evaluated

in triplicate by standard addition procedure with five known concentration levels from 50 to 200 % of assay analyte concentration (50, 75, 100, 150 and 200 μg $mL^{-1}$). For each concentration, three sets were prepared and injected in triplicate. The percent recoveries of added drug substance at each concentration were calculated.

### Solution Stability and Mobile Phase Stability

The solution stability of Zaleplon in the assay method was carried out by leaving the test solutions of samples in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed 6 h interval up to the study period against freshly prepared standard solution. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions 6 h interval up to 48 hrs. Mobile phase prepared was kept constant during the study period of mobile phase stability. The % RSD of assay of Zaleplon was calculated for the study period during mobile phase and solution stability experiments.

## RESULTS AND DISCUSSION

### Method Development and Optimization

The Zaleplon solutions were prepared in diluent at a concentration of 100 μg $mL^{-1}$ and scanned in UV-Visible spectrometer; the Zaleplon was having UV maxima at around 230 nm. Hence detection at 230 nm was selected for method development purpose.

The aim of the study was to develop optimized stability-indicating method to resolve the degradation product from the drug. So Water and methanol (65:35 v/v) was chosen for initial trial on a C18 stationary phase with a 25 cm length, 4.6 mm ID and 5μm particle size. Flow rate was 1.0 mL $min^{-1}$.When Zaleplon sample was injected, the symmetry of Zaleplon peak was good but the RT of the principal peak was too high. Similar results were obtained with 15 cm length, 4.6 mm ID & 5 micron particle size C-8 column.

Effect of solvent was studied by using different solvent. When acetonitrile is used instead of methanol, though there was considerable reduction in the principal peak RT, but when the stressed samples were injected under these conditions, the peak purity of the Zaleplon was not passing indicating Co-elution of the degraded impurities. So it was decided to transfer the method to UPLC system in order to achieve reasonable retention and also cost effective & time effective method.

In UPLC system again taken acetonitrile as the solvent & injected the Zaleplon 0.1 mg/ml solution using the Acquity UPLC® BEH C18 (100 x 2.1) mm with 1.7 μm particles with mobile phase water & acetonitrile in the ratio 65:35 v/v. But the principal peak was eluted too early. So the chromatographic conditions were chosen after the test of different mobile phase ratios but the condition was optimized by taking methanol as the solvent instead of acetonitrile by considering the cost effectiveness of the method. The mobile phase A consists of pure water. The mobile phase B consists of Methanol. With the mobile phase ratio of 75: 25 v/v. Flow rate of the mobile phase was 0.4 *mL* $min^{-1}$. The column temperature was maintained at 25°C and the detection was monitored at a wavelength of 230 nm. The injection volume was 2 μL. When Zaleplon stressed sample was injected under these conditions, there was some co-elution of the degraded impurity as observed by peak purity study. So the mobile phase ratio was again changed to 65: 35 v/v. to separate the degradation impurity.

The satisfactory chromatographic separation (retention time of Zaleplon is ~7 min and the resolution ($R_s$) between all the impurities was >2) was achieved on Acquity UPLC® BEH C18 (100 x 2.1) mm with 1.7 μm particles with mobile phase water & methanol in the ratio 65:35 v/v. The column temperature as maintained at 25°C and the detection was monitored at a wavelength of 230 nm.The injection volume was 2 μL. Methanol was used as diluent. In the optimized LC condition the Zaleplon RT were about ~7 min. The system suitability results were given in (TABLE 1) and the developed LC method was found to be specific for Zaleplon and its Degradants. Peak purity of stressed samples of Zaleplon was checked by using 2996 Photo diode array detector of Waters (PDA). The purity angle within the purity threshold limit obtained in all stressed samples demonstrates the analyte peak homogeneity. All stressed samples of Zaleplon (heat (100°C), acid hydrolysis (1N HCl), base hydrolysis (0.1N NaOH), water hydrolysis and oxidation (3 % $H_2O_2$)) were ana-

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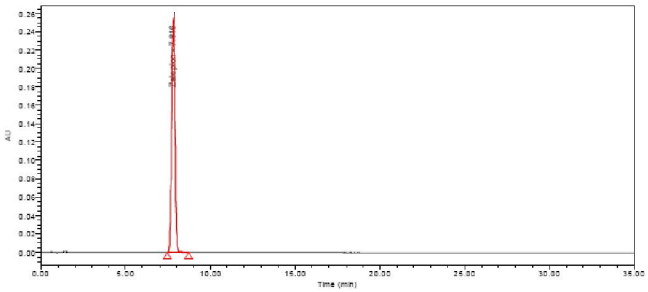
lyzed for extended run time of 60 min to check the late eluting degradants.The typical chromatogram of System suitability shown in Figure 2(a).

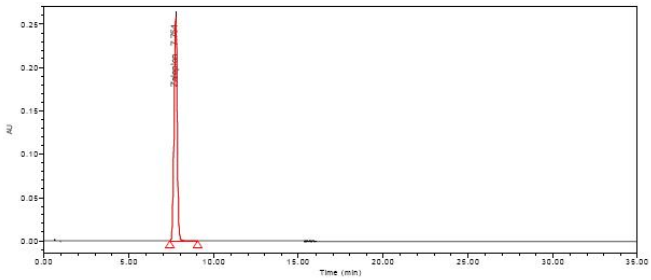
The proposed method was applied for the assay analysis of 3 different batches of Zaleplon. The typical chromatograms of assay are shown in Figure 2(b) .The mass balance (% of assay + % of degradation products + % of impurities) of stressed samples was close to 99.8 % (TABLE 2). The assay of Zaleplon is unaffected in the presence of its degradation products confirming the stability indicating power of the developed method.

**TABLE 1 : System Suitability report**

| Compound | Retention time | No of theoretical plates USP tangentmethod (*N*) | USP Tailing factor (*T*) | Purity Angle | Purity Threshold |
|---|---|---|---|---|---|
| Zaleplon | 7.8 | 7829 | 0.98 | 0.015 | 0.216 |



**A : Typical chromatograms of Zaleplon (System suitability)**



**B : Zaleplon batch sample at assay concentration (0.1 mg/ml)**

**Figure 2 : Typical chromatograms of Zaleplon (System suitability), and assayed sample (0.1mg/mL)**

**TABLE 2 : Summary of forced degradation results**

| Stress condition | Time | % Assay of stressed sample | % Mass Balance ( % of assay + % of degradation products + % of impurities) |
|---|---|---|---|
| Acid hydrolysis (1N HCl) (reflux at 80°C) | 8 h | 92.1 | 99.8 |
| Base hydrolysis (0.1N NaOH) (reflux at 80°C) | 2 h | 90.4 | 99.7 |
| Oxidation (3% $H_2O_2$) (reflux at (reflux at 80°C) | 3 h | 88.6 | 99.6 |
| Water hydrolysis (reflux at 100°C) | 2 h | 99.5 | 100 |
| Light (photolytic degradation) | 10 days | 99.8 | 100 |
| Thermal (100°C) | 10 days | 99.7 | 100 |

## Method validation

### Precision

The % RSD of assay of Zaleplon during assay method precision study was 0.2 % and confirming the good precision of the developed analytical method.

The % RSD of assay results obtained in intermediate precision study was within 0.3 % and, confirming the ruggedness of the method (TABLE 3).

**TABLE 3 : Results of Intermediate precision**

| S.No | Parameter | Variation | %RSD for Assay | % Assay |
|---|---|---|---|---|
| 2 | Different Column | (a) B.No: 001 | 0.4% | 99.7 |
| | | (b) B.No:002 | 0.5% | 99.6 |
| 3 | Different Analyst | (a) Analyst-1 | 0.5% | 99.8 |
| | | (b) Analyst-2 | 0.4% | 99.7 |
| 4 | Different Days (Interday precision) | (a) Day-1 | 0.4% | 99.7 |
| | | (b) Day-2 | 0.6% | 99.6 |
| | | (c) Day-3 | 0.5% | 99.8 |

## Linearity

Linear calibration plot obtained by the least square regression analysis for assay method was obtained over the calibration ranges tested, i.e. 25-200 μg $mL^{-1}$ and the correlation coefficient obtained was greater than 0.9997. The Slope and the Intercept value obtained from the linear regression graph is as shown in (TABLE 4). The result shows an excellent correlation existed between the peak area and concentration of the analyte in the range 50-200 % of analyte concentration.

**TABLE 4 : Results of Linearity study the drug substance**

| Concentration (μg) | Response |
|---|---|
| 25 | 858229 |
| 50 | 1749589 |
| 75 | 2540289 |
| 100 | 3379575 |
| 150 | 4225285 |
| 200 | 5062589 |
| Calibration Equation | y=34562.5x-47178.4 |
| Linearity Range | 25-200 % |
| Regression coefficient | 0.9997 |
| Slope | 34562.5 |
| Intercept | -47178.4 |

## Accuracy

The percentage recovery of Zaleplon in bulk drug samples ranged from 99.2 to 101.4% (TABLE 5) at each level of the determination, which indicated good accuracy of the method.

**TABLE 5 : Results of Accuracy study for drug substance**

| Added (μg) (*n*= 3) | Recovered (μg) | % Recovery | % RSD |
|---|---|---|---|
| 50 | 49.9 | 99.8 | 0.6 |
| 75 | 74.4 | 99.2 | 0.4 |
| 100 | 99.7 | 99.7 | 0.9 |
| 150 | 150.9 | 100.6 | 0.3 |
| 200 | 203 | 101.4 | 0.5 |

***n* =3, Number of determinations**

## Solution Stability and Mobile phase Stability

The % RSD of assay of Zaleplon during solution stability and mobile phase stability experiments was within 1.0. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay were stable up to the study period of 48 h.

## Results of Forced Degradation Studies

### Degradation Behavior

Stress studies on Zaleplon under different stress conditions suggested the following degradation behavior.

### Degradation in Acidic solution

The drug was exposed to 1N HCl at 80°C for 8 HRS. Zaleplon has shown significant sensitivity towards the treatment of 1 N HCl . The drug gradually undergone degradation with time in 1 N HCl and Prominent degradation was observed (~7.1 %) (Figure 3(a))

### Degradation in Basic solution

The drug was exposed to 0.1 N NaOH at 80 °C for 2hrs. Zaleplon has shown significant sensitivity towards the treatment of 0.1 N NaOH. The drug undergone degradation immediately in 0.1 N NaOH and prominent degradation was observed (~10.2 %) (Figure 3(b))

### Oxidative Conditions

The drug was exposed to 80°C for 3 hrs in 3% H2O2. Zaleplon has shown significant sensitivity towards the treatment of 3 % hydrogen peroxide and the drug gradually undergone oxidative Degradation with time in 3% hydrogen peroxide and prominent degradation was observed (~11.0 %) (Figure 3(c)). Purity plot obtained shows the peak homogeneity (Figure 3(d)).

### Degradation in Neutral (Water) solution

No major degradation products were observed after 48 h at room temperature. Degradation was not observed even when more stressed conditions were applied (reflux at 100°C).

### Photolytic Conditions

The drug was stable to the effect of photolysis. When the drug powder was exposed to light for an overall illumination of 1.2 million lux hours and an integrated near ultraviolet energy of 200-watt hours/square meter (w/mhr) (in photo stability chamber), no degradation was observed.

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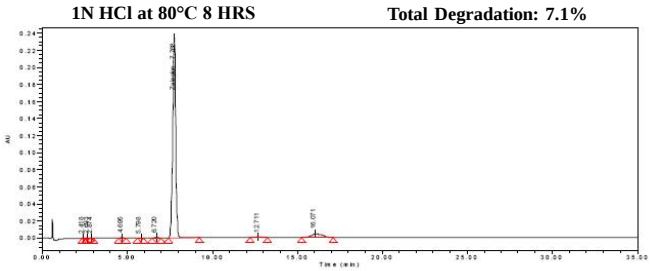
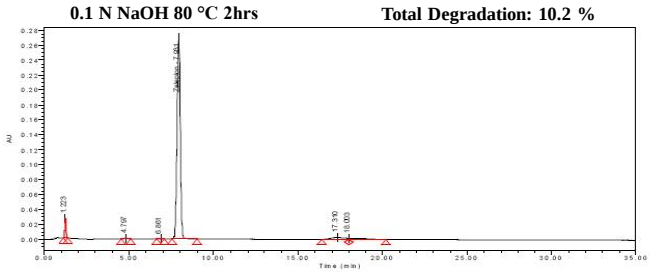
## Thermal Degradation

The drug was stable to the effect of temperature. When the drug powder was exposed to dry heat at 100°C for 10 days, no degradation was observed.

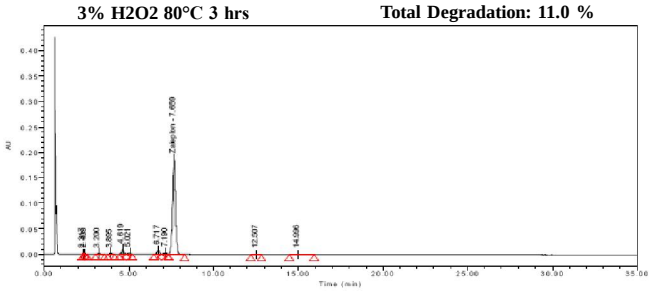
Peak purity test results derived from PDA detector, confirmed that the Zaleplon peak was homogeneous and pure in all the analyzed stress samples. No degradants were observed after 30 min in the extended runtime of 60 min of all the Zaleplon samples. The spectra of degraded sample matches with the standard spectra (Figure 3(e)).



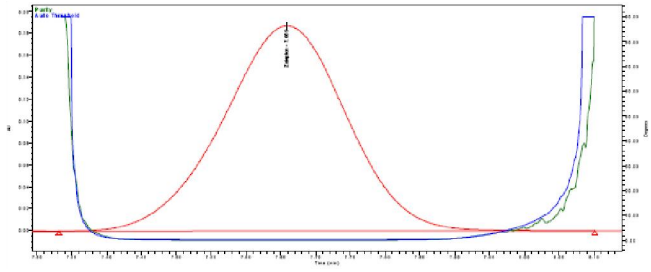

(a) : Zaleplon Acid Degradation sample



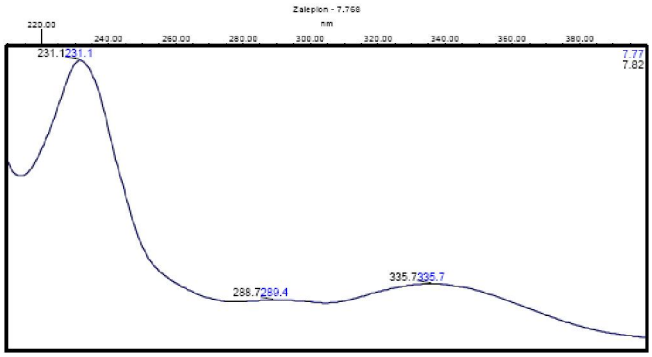

(b) : Zaleplon Base Degradation sample




(c) : Zaleplon Peroxide Degradation sample



(d) : Purity plot of Peroxide degradation




(e) : Acid degradation sample spectra matches with the standard spectra

**Figure 3 : Typical chromatograms of stressed Zaleplon with purity plot**

## Assay analysis

Assay analysis was performed for different batches of Zaleplon in bulk drug samples ($n$=3), with the analyte concentration of 100 µg $mL^{-1.}$ The assay results obtained for the three bulk drug samples were, ZLP/001(99.65 %), ZLP/002 (99.69%) and ZLP/003 (99.85%) depicted in (TABLE 6).

**TABLE 6 : Batch analysis for Zaleplon drug substance**

| Batch No: | Assay by HPLC |
|---|---|
| ZLP/001 | 99.65% |
| ZLP/002 | 99.69% |
| ZLP/003 | 99.85% |

## CONCLUSION

The Isocratic RP-UPLC method developed for quantitative assay of Zaleplon in bulk drug is precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of Zaleplon samples.

## ACKNOWLEDGEMENTS

The authors wish to thank the management of United States Pharmacopeia laboratory- India for supporting this work.

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