

**A sensitive and selective liquid chromatographic method for the determination of zolpidem from human plasma using fluorescence detector**

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Received: 13<sup>th</sup> September, 2008 ; Accepted: 18<sup>th</sup> September, 2008**ABSTRACT**

A simple, sensitive, selective LC method with a short run time (4.5 min) was developed and validated for the determination of Zolpidem from human plasma using fluorescence detector (excitation 254 nm and emission 390 nm). The samples were deproteinised using methanol. After deproteinization the analyte and the internal standard (Etodolac) were separated on a C<sub>18</sub> column using 5mM Potassium Dihydrogen Phosphate: Acetonitrile (65:35 v/v; pH 7.5 with triethylamine) as mobile phase. The analyte exhibited a linear range of 2.08-246.43 ngmL<sup>-1</sup>. Acceptable Precision and Accuracy were obtained for the concentrations over the standard curve range. The Intra-day Accuracy ranged between 91.25-111.21 % with a precision of 2.03-5.77%. The Inter-Day accuracy was between 95.39-111.21% with a precision of 2.13-3.58%. The validated method was successfully applied for the quantitation of Zolpidem from human plasma samples in a pharmacokinetic study. © 2008 Trade Science Inc. - INDIA

**KEYWORDS**

Zolpidem;  
Etodolac;  
Liquid chromatography (LC);  
Fluorescence detection (FLD);  
Pharmacokinetics.

**INTRODUCTION**

Zolpidem<sup>[1]</sup> (ZLP) which is N,N,6-trimethyl-2-(4-methylphenyl)-imidazo(1,2-a)pyridine-3-acetamide used for the short-term treatment of insomnia<sup>[2]</sup>, as well as some brain disorders. It is a short-acting nonbenzodiazepine hypnotic that potentiates gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter, by binding to benzodiazepine receptors which are located on the gamma-aminobutyric acid receptors. Zolpidem is absorbed rapidly and the absolute bioavailability of the drug is about 70%<sup>[3]</sup>. The drug is almost protein bound in plasma (92%). Elimination of Zolpidem is rapid, with a terminal elimination half-life of 2.1 h.

Several analytical methods have been reported for the quantitation of Zolpidem in human body fluids and organ samples including Capillary Electrophoresis<sup>[4]</sup>, Ra-

dioimmunoassay<sup>[5]</sup>, GC<sup>[6,7]</sup>, GC-MS<sup>[8,9]</sup>, LC-MS-MS<sup>[10,11]</sup> and HPLC<sup>[12-21]</sup>.

This paper describes a simple, sensitive, selective LC-FLD method with a short run time for the determination of ZLP in human plasma using Etodolac (ETD) as an internal standard. The quantitation limit is sufficiently to support pharmacokinetic and bioequivalence study and a very simple sample extraction procedure also allows high throughput analysis.

**EXPERIMENTAL****Chemicals and materials**

Methanol, Potassium dihydrogen phosphate and triethylamine were purchased from Merck (Merck, India). Water was deionised and purified by a milli-Q water purification system from Millipore (Bedford, MA,

USA). Blank CPDA (Citrate Phosphate Dextrose Adenine) plasma bags (six different lots) were obtained from J.V.P Blood Bank (Navi Mumbai-India)

### Instrument and chromatographic conditions

Agilent 1200 series HPLC (Waldbronn, Germany) equipped with isocratic pump (G1310A), autosampler (G1329A), Peltier cooler (G1330B), thermostatted column compartment (G1316A), variable wavelength detector (G1314B) and EZChrome Elite software (version-3.2.1) was used for the analysis. Analysis was performed on Gemini RP18e 5 cm  $\times$  4.6 mm i.d.; 5 $\mu$  particle size with 5mM potassium dihydrogen phosphate: Acetonitrile (65:35 v/v: pH 7.5 with triethylamine) as mobile phase. The column was maintained at 25°C. The flow rate was kept at 1.0 ml/min. The detection was carried out using fluorescence detector using 254nm as the excitation wavelength and 390 nm as the emission wavelength.

### Preparation of stock and working solutions

The stock solution of ZLP (1 mgmL<sup>-1</sup>) was prepared for calibration standards and quality control (QC) samples, by dissolving appropriate amount of the compound in methanol. This stock solution was subsequently serially diluted with mobile phase to obtain working solutions which when added to plasma would yield concentrations in the range 2.08-246.43 $\mu$ gmL<sup>-1</sup>. A 60 $\mu$ g mL<sup>-1</sup> working solution of ETD was prepared in mobile phase by dissolving 60 mg of the standard to 100 mL with methanol, and further diluting 5 mL of the same to 50 mL with mobile phase. All solutions were stored at 2-8°C

### Preparation of calibration standards, quality control samples

To 475 $\mu$ L of drug free plasma, 25 $\mu$ L of working solutions of ZLP (prepared as above) was added to yield final respective concentrations as: 2.08(LLOQ-lower limit of quantitation), 4.62, 9.24, 18.48, 37.96, 73.93, 123.22 and 246.43 ngmL<sup>-1</sup> of ZLP in plasma. Low QC (LQC)-6.81 ngmL<sup>-1</sup>, medium QC (MQC)-123.75 ngmL<sup>-1</sup> and high QC (HQC)-225.01 ngmL<sup>-1</sup> were also prepared in similar manner using the same stock solution.

### Method development

Zolpidem (pK<sub>a</sub>-6.2) being a weakly basic compound, a buffered mobile phase was chosen to maintain consistency in the retention time and selectivity. Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) was used as a buffer because potassium is a stronger counter ion than sodium and it provides improved results as compared to sodium. To have a good control on the pH of the mobile phase and to obtain a good peak shape the pH of the mobile phase was adjusted to 7.5.

Solvents like 10% PCA (perchloric acid) (1:1), Acetonitrile (1:2) and Methanol (1:2) were tried for protein precipitation. The recovery of Zolpidem was less than 25% with PCA. The peak shape of Zolpidem distorted in samples containing Acetonitrile over a period of time because the elution strength of the acetonitrile was substantially higher than that of the mobile phase. Methanol was found to be a suitable deproteinising agent because very good recovery was obtained. Also the peak shape was satisfactory.

### Sample preparation

To 0.5 mL of the calibration standards and QC samples taken in polypropylene tubes, 1.0 mL of methanol was added and vortexed for 3 mins. After centrifuging for 5 min at 12000 rpm and 4°C, 1.0 mL of the clear supernatant methanol layer was separated and transferred to a 1.5 ml HPLC vial and 50 $\mu$ L of the solution was injected in the HPLC.

### Method validation<sup>[22]</sup>

#### Selectivity and sensitivity

For specificity the six different lots of blank plasma were evaluated for the interference at retention time of ZLP and ETD. Selectivity was carried out to evaluate the ability of the method to selectively quantify the drug and the IS from other plasma components after extraction by analyzing the six blank plasma spiked with ZLP (LLOQ level) and ETD.

#### Calibration curve and lower limit of quantitation (LLOQ)

The linearity of the method was evaluated using freshly prepared spiked plasma samples in the concentration range of 2.08-250 ngmL<sup>-1</sup> using the method of least squares. Five such linearity curves were analyzed.

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Each calibration curve consisted of a blank sample, a zero sample (blank+IS) and eight calibrator concentrations. Samples were quantified using the ratio of peak area of analyte to that of IS. A weighted linear regression (1/x) was performed with nominal concentrations of calibration levels. Peak area ratio was plotted against plasma concentrations and standard curves were calculated by the equation:  $y = mx + c$ .

### Extraction efficiency

The extraction efficiency of ZLP was evaluated by comparing the mean peak response of the extracted LQC, MQC and HQC to the mean peak response of three unextracted standards. Similarly, the recovery of IS was evaluated by comparing the mean peak responses in the three quality control samples to mean peak responses of three unextracted standards of at the concentration of 60000 ng/mL.

### Precision and accuracy

Intra-day and Inter day accuracy and precision were evaluated from replicate analysis ( $n = 6$ ) of QC samples containing ZLP at different concentrations (low, medium and high) on the same day and on three separate occasions. QC samples were analysed against calibration curves. Each validation run consisted of a minimum of one set of calibration standards and six sets of QC samples at three concentrations.

### Stability

Evaluation of stability of samples was based on the comparison of stability samples against freshly prepared samples of the same concentration. % Difference between the back calculated concentrations obtained in the stability sample and freshly prepared sample was evaluated.

For Long Term stability (LTS), the samples were kept in deep freezer at  $-20 \pm 5^\circ\text{C}$  for 30 days and thereafter analyzed. Bench Top stability (BTS) was studied on samples kept at ambient temperature ( $20-30^\circ\text{C}$ ) for 8 hours. The processed samples were kept in the autosampler at  $7^\circ\text{C}$  for 24 hours and then injected to determine the autosampler stability (ATS). The freeze-thaw stability (FTS) (samples stored at  $-20 \pm 5^\circ\text{C}$ ), was studied after subjecting the samples to three freeze thaw cycles.

In order to determine the long term stability of ZLP

and ETD in solution, the working solutions were kept at  $2-8^\circ\text{C}$  for 6 days. Thereafter, the mean areas of ZLP and ETD from six replicate chromatographic runs of the stability solution were compared to that of mean area of freshly prepared solution of same concentration. The short term stability of the solution was also evaluated by keeping the working solutions at ambient temperature for 6 hours and then comparing the area of stability solution with that of freshly prepared solution.

## RESULTS AND DISCUSSION

Under these chromatographic conditions the peaks of ZLP and ETD eluted at about 3 minutes and 2 minutes respectively. The analysis time was 4.5 minutes. A typical HPLC chromatogram showing ZLP at LLOQ concentration is shown in figure 1.

### Selectivity and Sensitivity

No interfering peaks were observed at the retention times of either analyte or internal standard in six different lots of drug free human plasma samples used for analysis. The representative HPLC chromatogram of extracted blank plasma is shown in figure 2. The

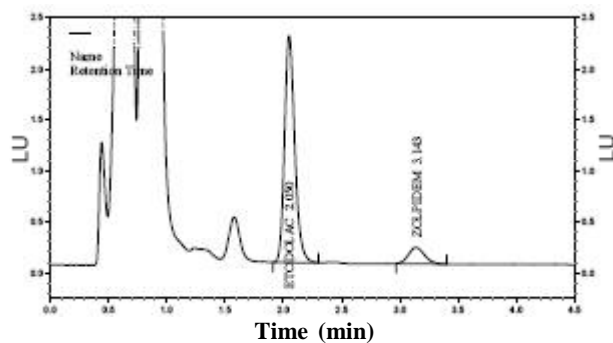


Figure 1: The representative HPLC chromatogram of ZLP at LLOQ concentration

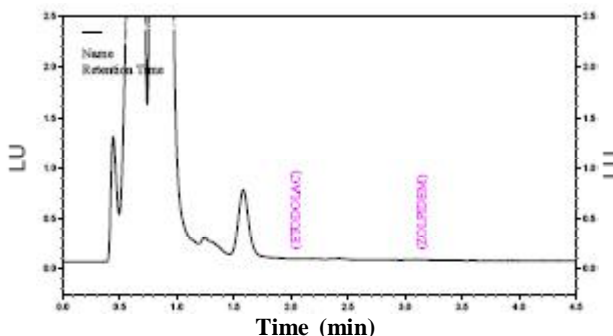


Figure 2: The representative HPLC chromatogram of extracted blank plasma

**TABLE 1: Summary of the results of five calibration curves for determination of Zolpidem in human plasma**

Concentration (ngmL <sup>-1</sup> )	Conc. Found (mean±SD, ngmL <sup>-1</sup> )	Mean accuracy(%)	Precision (%RSD)
2.08	2.13 ± 0.10	102.44	4.87
4.62	4.58 ± 0.07	99.12	1.42
9.24	9.20 ± 0.23	99.53	2.48
18.48	18.71 ± 0.53	101.23	2.84
36.96	37.66 ± 0.79	101.88	2.10
73.93	73.91 ± 1.37	99.97	1.86
123.22	114.30 ± 3.53	92.77	3.09
246.43	253.49 ± 3.42	102.86	1.35

SD-standard deviation; RSD-relative standard deviation

**TABLE 2: Summary of the intra and inter batch precision and accuracy of the method for the determination of Zolpidem in human plasma**

Concentration (ngmL <sup>-1</sup> )	Conc. Found (mean±SD, ngmL <sup>-1</sup> )	Mean accuracy(%)	Precision (%RSD)
<b>Intra day precision and accuracy results</b>			
6.81	7.25 ± 0.19	106.42	2.58
123.75	129.29 ± 7.46	104.47	5.77
225.01	248.66 ± 5.04	110.51	2.03
<b>Inter day precision and accuracy results</b>			
6.81	7.34 ± 0.16	107.76	2.13
123.75	129.84 ± 4.04	104.92	3.11
225.01	243.29 ± 8.70	108.12	3.58

**TABLE 3: The stability data of ZLP in human plasma**

Concentration (ngmL <sup>-1</sup> )	% Difference	
	LQC (6.81 ngmL <sup>-1</sup> )	HQC (225.01 ngmL <sup>-1</sup> )
Short term stability (8.0 hours, ambient temperature)	-3.35	0.74
Autosampler stability (24 hours)	4.17	4.24
Freeze thaw stability (3 cycles)	0.85	-0.53
Long term stability (30 days, -20°C)	-2.46	-0.24

**TABLE 4: Pharmacokinetic parameters of ZLP in 28 healthy subjects after a single 12.5 mg oral dose**

Parameters	Mean ± SD
C <sub>max</sub> (ngmL <sup>-1</sup> )	50.8 ± 23.66
AUC <sub>24h</sub>	230.3 ± 133.0
T <sub>max</sub> (h)	1.6 ± 0.7
t <sub>1/2</sub> (h)	2.5 ± 1.1

relative standard deviation for the six plasma samples spiked with ZLP at LLOQ concentration was 2.36% with accuracy ranging from 89.94-95.71%.

### Calibration curve and LLOQ

All calibration curves were found to be linear over the calibration range of 2.08-246.43 ngmL<sup>-1</sup>. The mean

equation of the calibration curve was  $y=19.931x+0.069$  with a correlation coefficient was 0.9971. The LLOQ was 2.08 ngmL<sup>-1</sup> with precision of 4.87% and accuracy of 102.44%. The results are presented in TABLE 1

### Extraction efficiency

The extraction efficiency of ZLP from human plasma at the concentrations of Low QC, MQC and High QC were found to be 95.49%, 93.25% and 95.85% with precision of 0.92%, 5.48% and 3.17%, respectively. The mean recovery for internal standard was 97.42%.

### Precision and accuracy

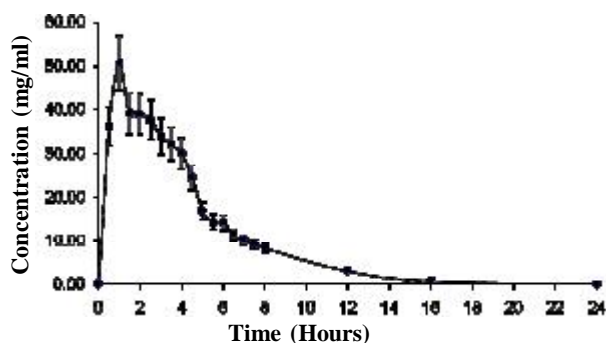
The intra-day accuracy ranged between 91.25% and 111.21 % with a precision of 2.03% to 5.77%. The inter-day accuracy was between 95.39% and 111.21% with a precision of 2.13% to 3.58%. The results are presented in TABLE 2.

### Stability

ZLP was found to be stable in human plasma for 30 days at -20°C (LTS), for 8 hours at ambient temperature (BTS), for 24 hours in autosampler (ATS), and for three freeze-thaw cycles (FTS). The stability data in tabulated in TABLE 3.

### Application to pharmacokinetic study

Figure 3 shows the time course of the mean plasma concentration of 28 healthy human subjects who received 12.5 mg Zolpidem formulation under fasting conditions. The study was conducted in accordance with guidelines laid down by the International Conference on Harmonisation and USFDA<sup>[23]</sup>. The pharmacokinetic data is tabulated in TABLE 4.



**Figure 3: Time course of the plasma ZLP concentration in healthy subjects after a single 12.5 mg oral dose. Each point represents the mean ± SD. (n=28)**

**Full Paper****CONCLUSION**

The proposed bioanalytical method is a single step process, very simple to perform. This method has also been found to be economic over all the previously reported methods because it uses inexpensive solvents and extractants. The short run time of this method facilitates a high throughput analysis which is best suited to monitor the plasma concentration in pharmacokinetic studies. The proposed method therefore can be used for routine drug monitoring also.

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