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Spectrochimica Acta Part A 68 (2007) 1220–1230
SPECTROCHIMICA ACTA PART A
www.elsevier.com/locate/saa

Application of spectrophotometric, densitometric, and HPLC techniques as stability indicating methods for determination of Zaleplon in pharmaceutical preparations

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Received 7 October 2006; received in revised form 14 January 2007; accepted 24 January 2007

Abstract

Spectrophotometric, spectrodensitometric and HPLC are stability indicating methods described for determination of Zaleplon in pure and dosage forms.

As Zaleplon is easily degradable, the proposed techniques in this manuscript are adopted for its determination in presence of its alkaline degradation product, namely *N*-[4-(3-cyano-pyrazolo[1,5a]pyridin-7-yl)-phenyl]-*N*-ethyl-acetamide. These approaches are successfully applied to quantify Zaleplon using the information included in the absorption spectra of appropriate solutions.

The second derivative (D_2) spectrophotometric method, allows determination of Zaleplon without interference of its degradate at 235.2 nm using 0.01N HCl as a solvent with obedience to Beer's law over a concentration range of 1–10 $\mu g\,ml^{-1}$ with mean percentage recovery $100.24 \pm 0.86\%$.

The first derivative of the ratio spectra (^1DD) based on the simultaneous use of (^1DD) and measurement at 241.8 nm using the same solvent and over the same concentration range as (D_2) spectrophotometric method, with mean percentage recovery $99.9 \pm 1.07\%$.

The spectrodensitometric analysis allows the separation and quantitation of Zaleplon from its degradate on silica gel plates using chloroform:acetone:ammonia solution (9:1:0.2 by volume) as a mobile phase. This method depends on quantitave densitometric evaluation of thin layer chromatogram of Zaleplon at 338 nm over a concentration range of 0.2–1 $\mu g\,band^{-1}$, with mean percentage recovery 99.73 ± 1.35.

Also a reversed-phase liquid chromatographic method using 5-C8 (22 cm × 4.6 mm i.d. 5 μm particle size) column was described and validated for quantitation of Zaleplon using acetonitrile:deionised water (35:65, v/v) as a mobile phase using Paracetamol as internal standard and a flow rate of 1.5 $ml\,min^{-1}$ with UV detection of the effluent at 232 nm at ambient temperature over a concentration range of 2–20 $\mu g\,ml^{-1}$ with mean percentage recovery $100.19 \pm 1.15\%$.

The insignificance difference of the proposed methods results with those of the reference one proved their accuracy and precision.

Keywords: Zaleplon; Derivative spectrophotometry; Derivative ratio spectrodensitometry; HPLC

1. Introduction

Zaleplon is *N*-[3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl) phenyl]-*N*-ethyl acetamide [1]. It is a pyrazolopyrimidin derivative. Although not structurally related to the benzodiazepines, Zaleplon acts through binding to the γ-amino butyric acid ($GABA_A$)-benzodiazepine receptor complex producing sedative and hypnotic effects similar to those of benzodiazepines [2–4]. It is reported to have relative selectivity for the Ω_1-subtype of benzodiazepines binding site [5]. The chemical structure, molecular weight and molecular formula of Zaleplon are illustrated in Fig. 1.

The literature survey reveals several methods for determination of Zaleplon in plasma, namely, liquid chromatography–electro spray ionisation–mass spectrometry assay, high-performance liquid chromatography, chemical ionisation–mass spectrometry [6–8], and RP-HPLC with fluorescence detection [9]. A spectrofluorimetric technique is used for its determination in micellar medium [10]. Zaleplon was found among drugs screened in hair and oral fluids using LC–MS [11,12]. It was quantitatively screened in blood as silylated derivative by gas chromatography–selected ion monitoring mass spectrometry and gas chromatography electron capture detection [13]. A fast gas chromatography–negative-ion chemical ionization

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doi:10.1016/j.saa.2007.01.024

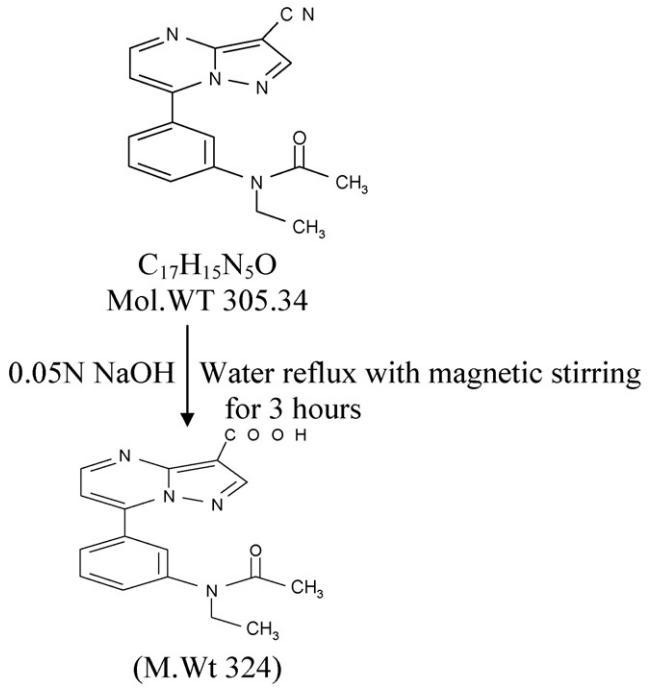

Fig. 1. The chemical structure, molecular weight and molecular formula of Zaleplon followed by the degradation pattern of Zaleplon in 0.05N NaOH.

mass spectrometry with micro scale volume samples preparation method was described for its determination in blood [14]. It was determined in capsules by voltammetric method [15]. A capillary electrophoresis with laser-induced fluorescence detection and liquid chromatography–mass spectrometry techniques were adopted for separation and identification of its metabolites in human urine [16].

The above literature revealed that up to the present time nothing has been published concerning the proposed methods for determination of Zaleplon in presence of its alkaline degradation product.

The proposed procedures were successfully applied to the routine and quality control analysis of Zaleplon in its pharmaceutical preparation.

2. Experimental

2.1. Apparatus

1- Shimadzu UV–vis 1601 PC spectrophotometer with 1 cm quartez cuvettes for spectrophotometric determinations (Kyoto, Japan).
   The following requirements are taken into consideration:
   - Scan speed: fast.
   - $\Delta\lambda$: 4 (for both D_2 and ^{1}DD methods).
   - Scaling factor: 100 (for D_2 method) and 10 (for ^{1}DD method).
2- UV lamp with short wavelength 254 nm (USA).
3- TLC Scanner 3 densitometer (Camage, Muttenz, Switzerland).
   The following requirements are taken into consideration:
   - Slite dimensions = 6.00 × 0.445, Micro.
   - Scanning speed = 20 mm s^{-1}.
   - Data resolution = 100 μm $step^{-1}$.
4- Sample applicator for TLC linomat IV with 100 μl syringe (Camage, Muttenz, Switzerland).
5- TLC plates (20 cm × 20 cm) coated with silica gel 60 F254 (Merck KgaA, Darmstad, Germany).
6- The HPLC system consisted of a Perkin Elmer system equipped with series 200 auto sampler, series 200 lc pump, series 200 UV/vis detector. The stationary phase was 5-C_8 (22 cm × 4.6 mm i.d. 5 μm particle size) column using acetonitrile:deionised water (35:65, v/v) as a mobile phase.

2.2. Materials

2.2.1. Authentic samples

1- Zaleplon (Batch No. 0550407) was kindly supplied by The Egyptian Co. for Pharmaceutical and Chemical Industries. S.A.E (EPCI), Industrial Zone, Bayad El-Arab, Beni Suef, Egypt. Its purity was reported to be 98.5% according to the company analysis certificate.
2- Paracetamol (Batch No. 34055) was kindly supplied by Memphis Co. for Pharm and Chemical Ind., Cairo, Egypt. Its purity reported to be 99% according to the company analysis certificate.

2.2.2. Dosage form

1- Siesta® capsules (Batch No. 50507), labeled to contain 10 mg Zaleplon is from SIGMA For Al Andalous Medical Company, Cairo, Egypt.
2- Zalocid® capsules (Batch No. 044060609/3), labeled to contain 10 mg Zaleplon is from The Egyptian Co. for Pharmaceutical and Chemical Industries. S.A.E. (EPCI).

2.2.3. Chemicals and solvents

1- Chloroform, hydrochloric acid, acetone, ammonia solution, NaOH and methanol all are from (El NASR Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).
2- Acetonitrile HPLC grade (SDS, France).
3- Deionised water (SEDICO pharmaceuticals Co., Cairo, Egypt).

N.B.: All the chemicals were of analytical grade. The solvents used for the spectrophotometric and spectrodensitometric methods were of spectrophotometric grade and those used for HPLC were of HPLC grade.

2.2.4. Degraded samples

2.2.4.1. Preparation of pure degraded sample. 0.5 g Zaleplon powder was transferred to 250 ml stoppered flask, water refluxed with 100 ml 0.05N NaOH with magnetic stirring for 3 h. The solution was cooled, the collected precipitate contains a mixture of Zaleplon and its degradate, from which the degradate was separated in pure form by preparative normal phase column chromatography using silica gel as a stationary phase and chloro-

form as a mobile phase with gradient increase in polarity by 1% acetone (using 2 ml ammonia as constant volume) each 100 ml addition. Fractions were collected each 10 ml and the degradate was identified by TLC using chloroform:acetone:ammonia (9:1:0.2, v/v/v) as mobile phase. Similar fractions were collected together and allowed to dry at room temperature until constant weight. The dry powder was identified by IR and the mass spectrometry.

3. Standard preparations

3.1. Stock solutions

3.1.1. For D_2, ^1DD spectrophotometric and spectrodensitometric methods

Stock solutions for each of Zaleplon and the degradate are prepared by weighing accurately 0.1 g of pure powder of each, transfer into two separate 100-ml volumetric flasks, add 75 ml methanol, shake well then complete to the mark with the same solvent to get 1000 $\mu g\,ml^{-1}$ stock solution for each.

3.1.2. For HPLC method

For Zaleplon, the degradate and internal standard (Paracetamol) were prepared as above but using acetonitrile:deionised water (35:65, v/v) as a solvent.

3.2. Working solutions

3.2.1. For D_2 and ^1DD spectrophotometric methods

Ten millilitres of each of Zaleplon and its degradate were separately transferred into two separate 100-ml volumetric flasks, completed to the mark with 0.01N HCl to get 100 $\mu g\,ml^{-1}$ working solutions.

3.2.2. For spectrodensitometric method

For each of Zaleplon and the degradate working solutions proceed as above but using methanol as a solvent.

3.2.3. For HPLC method

For Zaleplon, Paracetamol and the degradate proceed as above but using acetonitrile:water (35:65, v/v) as a solvent.

4. Procedures

4.1. D_2 and ^1DD spectrophotometric methods

4.1.1. Linearity and construction of calibration curve

The D_2 absorption spectra of Zaleplon in the range of (1–10 $\mu g\,ml^{-1}$) were recorded using 0.01N HCl as a blank (for D_2 spectrophotometric method). The absorption spectra of Zaleplon in the same range were divided by the absorption spectrum of 6 $\mu g\,ml^{-1}$ of the degradate (the divisor). The obtained ratio spectra were differentiated with respect to wavelength (for ^1DD method). The amplitude values at 235.2 and 241.8 nm were measured for both D_2 and ^1DD, respectively. The calibration graphs were then plotted, and regression equations were computed.

4.1.2. Assay of laboratory prepared mixtures for D_2 and ^1DD spectrophotometric methods

The D_2 and the ratio spectra of first derivative spectrophotometric curves of different laboratory prepared mixtures containing different ratios of Zaleplon and its degradate were recorded. The peak amplitudes at 235.2 and 241.8 nm were recorded for D_2 and ^1DD, respectively. The concentration of Zaleplon was calculated from the corresponding regression equations.

4.2. Spectrodensitometric method

4.2.1. Linearity and construction of calibration curve

Accurately measured aliquots equivalent to (0.2–1 μg) of Zaleplon working solution (100 $\mu g\,ml^{-1}$) were applied to a thin layer chromatographic plate (20 × 20) as a band using the Camage TLC sampler. Leave a space of 1 cm between each band and 1.5 cm from the bottom edge of the plate. Develop the plate in a chromatographic tank previously saturated for an hour with the developing mobile phase, chloroform:acetone:ammonia (9:1:0.2, v/v/v) by ascending chromatography at room temperature. Zaleplon bands were scanned at 338 nm. The calibration curve was constructed by plotting the peak height/1000 versus the corresponding concentration and the regression equation was developed.

4.2.2. Analysis of laboratory prepared mixtures

The peak heights of different laboratory prepared mixtures containing different ratios of Zaleplon and the degradate were measured and the concentration of Zaleplon in each mixture was obtained by applying in the corresponding regression equation.

4.3. HPLC method

4.3.1. Linearity and construction of calibration curve

Aliquots equivalent to (2–20 μg) of Zaleplon working solution (100 $\mu g\,ml^{-1}$) were separately transferred into a series of 10-ml volumetric flasks, 1.5 ml of Paracetamol working solution (100 $\mu g\,ml^{-1}$) was mixed with each one then completed to the volume with acetonitrile:deionised water (36:65, v/v). Triplicate 20 $\mu g\,ml^{-1}$ injection were made for each concentration. Chromatograms were recorded under the following instrumental parameters: flow rate was 1.5 ml min^{-1} at ambient temperature and the effluent was monitored at 232 nm. The separation was done on C8 column using acetonitrile:deionised water (36:65, v/v) as a mobile phase. The peak areas of Zaleplon and Paracetamol were recorded and the peak area ratio was calculated for each concentration. The calibration curve relating the obtained peak area ratio to corresponding concentration was constructed and the regression equation was then computed.

4.3.2. Analysis of laboratory prepared mixtures

The chromatographic conditions used under linearity and construction of calibration curve were applied for different laboratory prepared mixtures containing different ratios of Zaleplon and the degradate. The peak area ratio of Zaleplon and

Paracetamol was calculated for each mixture, Zaleplon concentration was calculated by substituting in the regression equation.

4.4. Application to pharmaceutical formulation

The content of 20 capsules of each of Siesta® capsules and Zalocid® capsules were separately emptied and mixed well, an amount of the powder of each equivalent to 0.1 g of Zaleplon was accurately weighed into 100-ml volumetric flask, 75 ml of appropriate solvent added, stirred magnetically for about 30 min, cooled well then completed to the volume to get 1000 μg ml^{-1} stock solution then filtered. Ten millilitres of the stock solution was accurately transferred into 100-ml volumetric flask, completed to the volume to get 100 μg ml^{-1} working solution, then the procedure detailed under linearity and construction of calibration curve for each method was followed.

When carrying out the standard addition technique, the powder content of the capsules and that of pure Zaleplon were mixed well together before proceeding in the above mentioned procedure.

5. Results and discussion

The high susceptibility of Zaleplon to hydrolysis makes its analysis in presence of its alkaline degradation product an analytical task of potential.

Upon degradation with sodium hydroxide, the separated degradate was confirmed using the IR and mass spectra analysis. The IR spectrum of the degradate showed that the peak of carbonyl group present in the parent compound was shifted from 1651 to 1660 cm^{-1} as broad peak indicating the presence of two carbonyl groups in the degradate. Peak of cyanide group which appeared in the drug at about 2231 cm^{-1} disappeared and a new one at 3399 cm^{-1} appeared indicating the presence of hydroxyl group. Presence of peak at 2982 cm^{-1} (of aliphatic protons of amide) in both drug and degradate indicated that the amide group was unaffected by the used hydrolysis conditions. The mass spectra confirm these claims where the base peak for each compound corresponds to its molecular weight (Fig. 2). On the other hand, hydrolsis using 0.05N HCl for 30 min was checked using TLC which showed that Zaleplon degraded completely giving three spots of minor components in addition to the major degradate. This caused great difficulty in separating and identifying these degradation products. The same results obtained on oxidation with hydrogen peroxide.

New methods for simultaneous determination of two or more compounds in the same sample without previous chemical separation are always of interest.

Derivative spectrophotometry offers greater selectivity than does the normal spectrophotometry, as it decrease spectral overlap and allows better resolution [17].

The ^{1}DD spectrophotometric method has an advantage that the measurements are carried out at peaks, hence there is a potential for greater sensitivity and accuracy [18]. The main disadvantage of zero crossing method in derivative spectrophotometry is the risk of small drifts of the working wavelengths, which may not fall in correspondence of peaks of the derivative spectrum. This may be dangerous when the slope of the spectrum is very high with consequent loss of accuracy and precession [19]. In the present case, the above circumstances did not occur.

The spectrodensitometric and HPLC methods were evaluated to improve the selectivity and sensitivity of Zaleplon analysis in presence of its degradation product.

Few procedures were found in the literature for determination of Zaleplon in absence of its degradation product. The proposed methods are devoted for the analysis of Zaleplon alone or in presence of its degradation product.

5.1. D_2 spectrophotometric method

The zero-order absorption spectra of Zaleplon and its degradate showed a serious overlapping (Fig. 3), that did not allow the use of direct spectrophotometric analysis of Zaleplon in presence of its degradate.

Derivative spectrophotometry has been applied extensively to the simultaneous determination of substances with overlapping spectra, which is frequently made on the basis of zero-crossing measurements.

Different solvents were tried to resolve this overlapping, e.g. methanol, absolute ethanol, acetonitrile, ethyl acetate, 0.01N NaOH and 0.01N HCl. In each of these cases the first, second and third derivatives were checked. By applying D_2 technique zero crossing point for Zaleplon with the degradate was shown at 235.2 nm (Fig. 4).

A linear correlation was obtained between the peak amplitude and the concentration in the range of 1–10 μg ml^{-1} from which the linear regression equation was computed and found to be:

$$Y = 0.0749C + 0.0025, \quad r = 0.9999$$

where Y is the peak amplitude at 235.2 nm, C the concentration in μg ml^{-1} and r is the correlation coefficient.

5.1.1. Optimization of D_2 spectrophotometric method

To optimize D_2 spectrophotometric method it was necessary to test the influence of different variables. Different smoothing factors ($\Delta\lambda$) values given by the program (2, 4 and 8) were tried where a smoothing factor = 4 showed a suitable signal to noise ratio and the spectra showed good resolution. Different scaling factor values (10, 100, and 1000) were tried where scaling factor 100 was suitable to enlarge the signal of Zaleplon to facilitate its measurement and to diminish error in reading signal.

This method is valid for determination of Zaleplon in presence of up to 60% of the degradate without interference with mean percentage recoveries of 101.29 ± 1.19% (Table 1).

Also the method was applied to assay Zaleplon in its pharmaceutical formulations, and its validity was further assessed by applying the standard addition technique (Table 3).

D_3 spectrophotometric method can also be used to resolve spectral overlapping of the absorption spectra of Zaleplon and its degradate at 226.4 nm ($\Delta\lambda = 8$, scaling factor = 100) (Fig. 5), but it was less selective than using D_2 spectrophotometric method (Table 2).

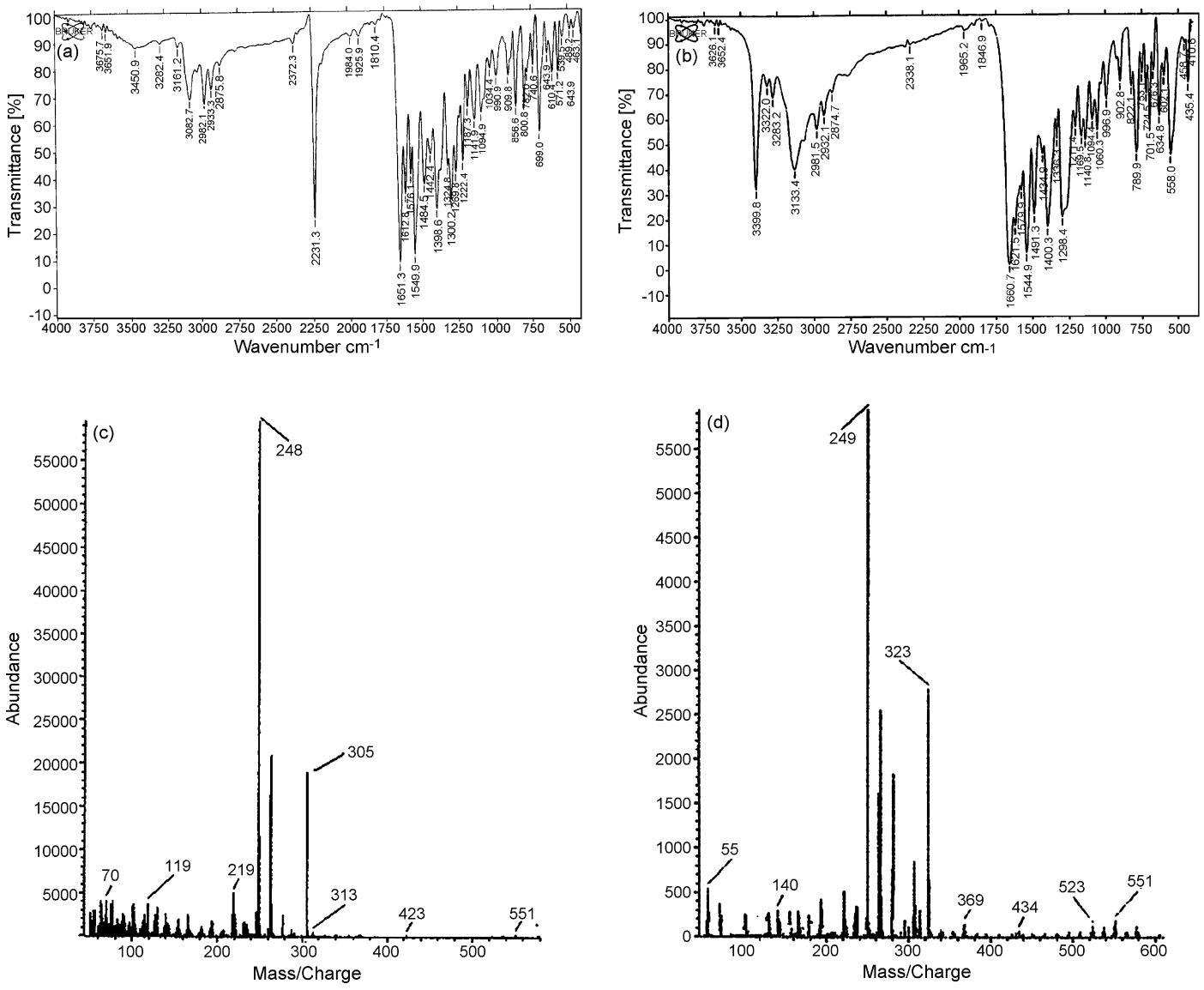

Fig. 2. IR and mass spectra of Zaleplon and its degradation product. (a) IR of Zaleplon, (b) IR of the degradate, (c) mass spectra of Zaleplon, and (d) mass spectra of the degradate.

5.2. ^{1}DD spectrophotometric method

The proposed technique is based on the use of first derivative of the ratio spectra for resolving binary mixtures [17].

To solve the problem of overlapping absorption spectra of Zaleplon and its degradate, Zaleplon can be assayed by dividing the absorption spectra of its different concentrations in the range (1–10 μg ml^{-1}) by the absorption spectrum of the degradate (6 μg ml^{-1}), the obtained ratio spectra were differ-

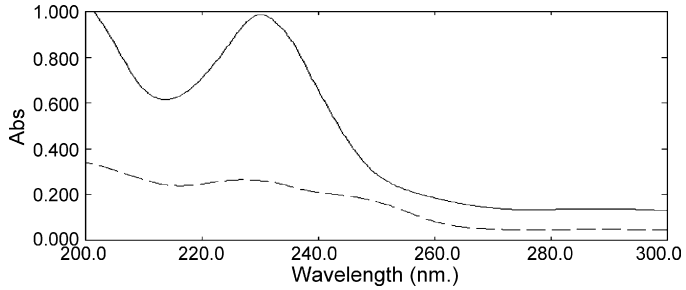

Fig. 3. Zero-order spectra of 8 μg ml^{-1} of Zaleplon (—) and 4 μg ml^{-1} of the degragate (- - -) in 0.01N HCl.

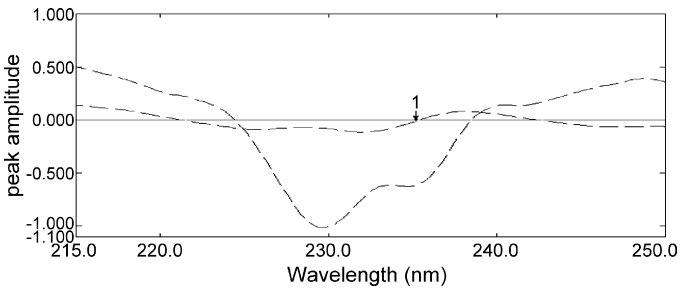

Fig. 4. The second derivative spectra of 8 μg ml^{-1} of Zaleplon and 4 μg ml^{-1} of the degradate (Spectrum indicated by arrow) in 0.01N HCl.

Table 1
Spectrophotometric determination of laboratory prepared mixtures for Zaleplon in presence of its degradation product by the second derivative (D_2) at 235.2 nm and the first derivative of ratio spectra (^{1}DD) at 241.8 nm methods

Mixture no.	Zaleplon %	Taken ($\mu g\,ml^{-1}$)	Second derivative (D_2 method)		First derivative of ratio spectra (^{1}DD method)	
			Found[a] ($\mu g\,ml^{-1}$)	Found %	Found[a] ($\mu g\,ml^{-1}$)	Found %
1	90	5.40	5.39	99.82	5.49	101.76
2	80	4.80	4.91	102.29	4.89	101.88
3	70	4.20	4.28	101.91	4.26	101.43
4	60	3.60	3.60	100.00	3.60	100.00
5	50	3.00	3.08	102.67	3.01	100.33
6	40	2.40	2.43	101.25	2.41	100.42
7	30	1.80			1.81	100.56
8	20	1.20			1.19	99.17
Mean ± S.D.				101.29 ± 1.19		100.69 ± 0.93

[a] Average of three experiments.

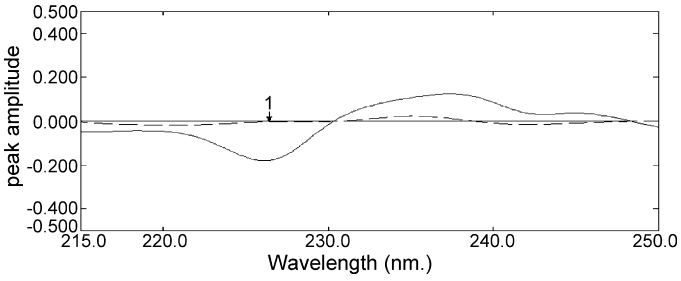

Fig. 5. The third derivative spectra of 8 $\mu g\,ml^{-1}$ of Zaleplon (—) and 4 $\mu g\,ml^{-1}$ of the degradate (···) in 0.01N HCl.

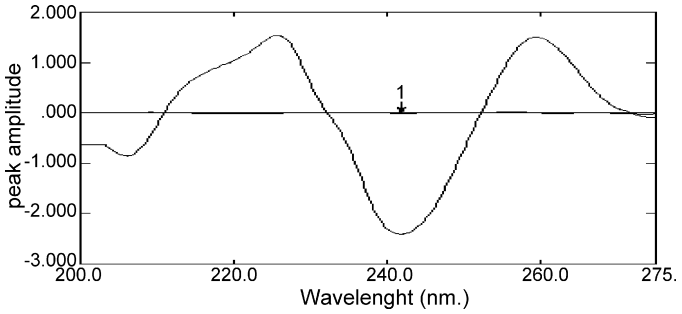

Fig. 6. First derivative of ratio spectra of 10 $\mu g\,ml^{-1}$ of Zaleplon (—) and 8 $\mu g\,ml^{-1}$ of the degradate (- - -) in 0.01N HCl.

entiated with respect to wavelength ($\Delta\lambda = 4$, scaling factor = 10) (Fig. 6).

DD_1 values showed good linearity and accuracy at $\lambda = 241.8$ nm. The linear regression equation was found to be:

$$Y = 0.2974C + 0.0248, \quad r = 0.9999$$

where Y is the peak amplitude at 241.8 nm, C the concentration in $\mu g\,ml^{-1}$ and r is the correlation coefficient.

5.2.1. Optimization of ^{1}DD spectrophotometric method

To optimize this method, it was necessary to test the influence of variables:

a- The divisor concentration: different concentrations of the degradate were tested, but concentration of (6 $\mu g\,ml^{-1}$) give the best results recording to sensitivity and selectivity.

b- Different smoothing factors ($\Delta\lambda$) values were tried where a smoothing factor = 4 showed a suitable signal to noise ratio and the spectra showed good resolution. Different scaling factor values were tried where scaling factor 10 was suitable to enlarge the signal of Zaleplon to facilitate its measurement and to diminish error in reading signal.

Table 2
Laboratory prepared mixtures for determination of Zaleplon in presence of its degradation product by the second derivative D_2, third derivative (D_3) and the first derivative of ratio spectra (^{1}DD) (at different wave lengths) spectrophotometric methods

Zaleplon % in the mixture	Second derivative (D_2) at 235.2 nm	Third derivative (D_3) at 226.4 nm	First derivative of ratio spectra (^{1}DD)		
			At 259.6 nm	At 241.8 nm	At 225.4 nm
Found %					
90	99.82	100.94	99.78	101.76	100.11
80	102.29	101.10	102.27	101.88	99.71
70	101.91	102.26	99.48	101.43	100.95
60	100.00	102.31	101.03	100.00	99.47
50	102.67	106.10[a]	99.87	100.33	101.77
40	101.25		107.96[a]	100.42	99.58
30	109.44[a]			100.56	100.06
20				99.17	90.92[a]
Mean ± S.D.	101.29 ± 1.19	101.65 ± 0.73	100.49 ± 1.16	100.69 ± 0.93	100.24 ± 0.84

[a] Using the rejection rule.

Table 3
Application of standard addition technique to the analysis of Zaleplon in capsules by second derivative (D_2) at 235.2 nm and the first derivative of ratio spectra (^{1}DD) at 241.8 nm

Taken ($\mu g\,ml^{-1}$)	Second derivative (D_2) method method		First derivative of ratio spectra (^{1}DD method)		Pure added ($\mu g\,ml^{-1}$)	Second derivative (D_2)		First derivative of ratio spectra (^{1}DD)	
	Found[a] ($\mu g\,ml^{-1}$)	Found (%)	Found[a] ($\mu g\,ml^{-1}$)	Found (%)		Pure found[b] ($\mu g\,ml^{-1}$)	Recovery %	Pure found[b] ($\mu g\,ml^{-1}$)	Recovery %
Siesta® capsules (Batch No. 50507)									
5.00					2.00	1.98	99.00	1.96	98.00
					3.00	2.94	98.00	3.02	100.67
					4.00	4.01	100.25	3.95	98.75
	4.86	97.20	4.87	97.40	5.00	5.09	101.80	5.05	101.00
Mean ± S.D.							99.76 ± 1.64		99.61 ± 1.46
Zalocid® capsules (Batch No. 044060609/3									
5.00					2.000	2.02	101.00	1.98	99.00
					3.000	2.99	99.67	3.06	102.00
					4.000	3.97	99.25	4.00	100.00
	4.80	96.00	4.83	96.60	5.000	5.08	101.60	5.00	100.00
Mean ± S.D.							100.38 ± 1.10		100.25 ± 1.26

[a] Average of six experiments.
[b] Average of three experiments.

Results obtained by the proposed technique showed that the concentration of Zaleplon can be determined in the presence of up to 80% of the degradate in the laboratory prepared mixtures with mean percentage recovery 100.69 ± 0.93% (Table 1).

This method was successfully applied to the determination of Zaleplon in pharmaceutical formulations and applying the standard addition technique assessed the validity of this method (Table 3).

^{1}DD can also be determined at 225.6 and 259.6 nm (Fig. 6), where the ^{1}DD value showed good linearity and accuracy but of little selectivity than at 241.8 nm (Table 2).

5.3. Spectrodensitometric method

This technique offers a simple way to quantify directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing the peak height of the unknown band to a standard curve from reference materials chromatographed simultaneously under the same condition [20].

The method is based on the difference in the R_f values of Zaleplon ($R_f = 0.447$) and the degradate ($R_f = 0.145$) (Fig. 7).

The calibration curve was constructed by plotting the peak height (1/1000) versus the concentration in the range of 0.2–1 $\mu g\,band^{-1}$, Zaleplon concentration was calculated from the following regression equation:

$$Y = 0.2174X + 0.0504, \quad r = 0.9996$$

where Y is the peak height (1/1000), X the concentration in $\mu g\,band^{-1}$ and r is the correlation coefficient.

5.3.1. Optimization of spectrodensitometric method

Studying of the optimum parameters for maximum separation was carried out by trying different developing systems with different ratios, but complete separation of Zaleplon and its degradate was achieved by using chloroform:acetone:ammonia (9:1:0.20, v/v/v).

Also different scanning wavelengths were tested, but the best sensitivity obtained when Zaleplon was scanned at 338 nm.

Results obtained in Table 4 showed that this method is selective, valid and applicable for determination of Zaleplon in presence of up to 90% of its degradate with mean percentage recovery 101.39 ± 1.53%.

The validity of the method was further assessed by applying the standard addition technique (Table 5).

5.4. HPLC method

A validated isocratic RP-HPLC method with UV detection was developed for the quantitation of Zaleplon in presence of its degradation product.

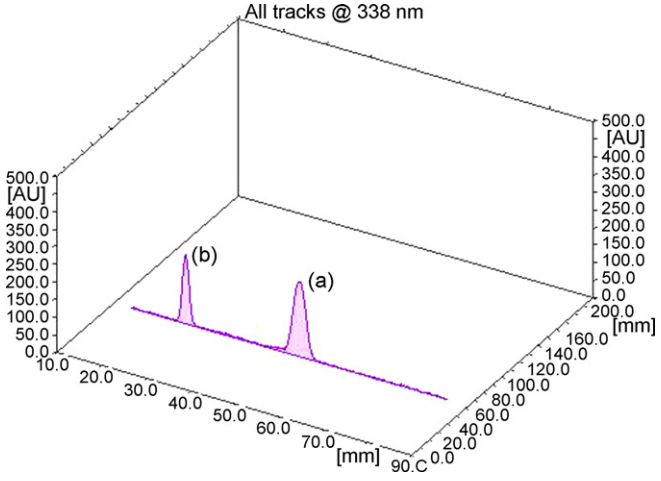

Fig. 7. 3D diagram showing an example of separated mixture of Zaleplon (a) and the degradate (b) in the spectrodensitometric method.

Table 4
Laboratory prepared mixtures for determination of Zaleplon in presence of its degradate by spectrodensitometric method at 338 nm

Mixture no.	Zaleplon %	Taken ($\mu g\,band^{-1}$)	Found[a] ($\mu g\,band^{-1}$)	Found %
1	90	0.54	0.55	101.85
2	80	0.32	0.32	100.00
3	70	0.70	0.69	98.57
4	60	0.48	0.49	102.08
5	50	0.60	0.62	103.33
6	40	0.56	0.57	101.79
7	30	0.42	0.43	102.38
8	20	0.40	0.41	102.5
9	10	0.20	0.20	100.00
Mean ± S.D.				101.39 ± 1.53

[a] Average of three experiments.

It depends on the chromatographic separation of the drug and its degradate using 5-C8 (22 cm × 4.6 mm i.d. 5 μm particle size) column and acetonitrile:water (35:65, v/v) as a mobile phase with UV detection at 232 nm. This method has the advantage of using an internal standard (Paracetamol), which compensates for any error that may occur due to baseline drift or fluctuations in readings of UV detectors.

A linear correlation was obtained between the peak area ratio and Zaleplon concentration in the range of 2–20 $\mu g\,ml^{-1}$. The regression equation was calculated and found to be:

$$Y = 0.162X - 0.0032, \quad r = 0.9998$$

where Y is the peak area ratio (of Zaleplon and Paracetamol), X the concentration in $\mu g\,ml^{-1}$ and r is the correlation coefficient.

5.4.1. Optimization of HPLC method

To optimize the proposed HPLC method, all of the experimental conditions were investigated. The stationary phase choice was selected to be the reversed-phase over the normal-phase separation due to the drawbacks of the normal phase, e.g. hydration of silica with water which can cause peak tailing. The optimum tailing factor was obtained when using C_8 column than that of C_{18} column, although they have the same sensitivity and selectivity.

Concerning the mobile phase, different systems were tried for chromatographic separation of the drug from its degradate by combining homogenous design and solvent polarity optimization. The best resolution was achieved when using a mobile phase consisting of acetonitrile:water (35:65, v/v).

Also the effect of pH was studied by using buffers of different pH values. It was found that changing the pH does not affect the peak shape.

Finally, a satisfactory separation was obtained by using acetonitrile:water (35:65, v/v) as a mobile phase, maintaining the flow rate of 1.5 $ml\,min^{-1}$ with UV detection at 232 nm.

Paracetamol was used as internal standard, where the retention time of Paracetamol was 1.69 min, the degradate was 2.5 min and Zaleplon was 4.06 min, with no interference among the three peaks. Typical chromatogram is shown in Fig. 8.

The efficiency of the proposed method as stability indicating one was assessed by applying analysis of laboratory prepared mixtures containing different ratios of intact Zaleplon and its degradation product, where Zaleplon could be determined in presence of up to 90% of the degradation products without interference, with mean percentage recoveries of 101.47 ± 0.83% (Table 6).

This method was applied to assay Zaleplon in its in dosage forms. The validity of the method was further assessed by applying the standard addition technique (Table 7).

5.5. Method validation

(a) Linearity:

The linearity of the proposed methods was evaluated by analyzing eight concentrations ranging between 1 and 10 $\mu g\,ml^{-1}$ (for both D_2 and ^1DD spectrophotometric methods), six concentrations ranging between 0.2 and 1 $\mu g\,band^{-1}$ (for the spectrodensitometric method), and nine concentrations ranging between 2 and 20 $\mu g\,ml^{-1}$ (for HPLC method). Each concentration was repeated three

Table 5
Application of standard addition technique to the analysis of Zaleplon in capsules by the spectrodensitometric method at 338 nm

Taken ($\mu g\,band^{-1}$)	Found[a] ($\mu g\,band^{-1}$)	Found %	Pure added ($\mu g\,band^{-1}$)	Pure found[b] ($\mu g\,band^{-1}$)	Recovery %
Siesta® capsules (Batch No. 50507)					
0.40	0.39	98.00	0.20	0.20	100.00
			0.30	0.30	100.00
			0.40	0.41	102.50
			0.50	0.50	100.00
Mean ± S.D.					100.63 ± 1.25
Zalocid® capsules (Batch No. 044060609/3)					
0.40	0.39	97.00	0.20	0.20	100.00
			0.30	0.30	100.00
			0.40	0.40	100.00
			0.50	0.51	102.00
Mean ± S.D.					100.50 ± 1.00

[a] Average of six experiments.
[b] Average of three experiments.

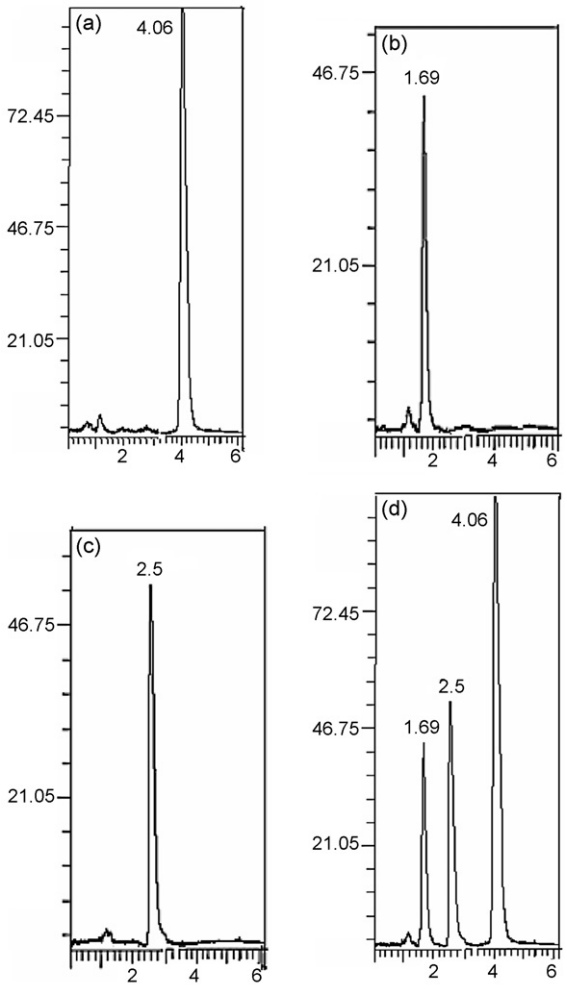

Fig. 8. HPLC chromatograms of (a) Zaleplon, (b) Paracetamol, (c) the degradate and (d) mixture of a, b and c. At 232 nm using acetonitrile:deionised water (35:65, v/v) as a mobile phase and a flow rate of 1.5 ml min^{-1} using Paracetamol as internal standard.

Table 6
Laboratory prepared mixtures for determination of Zaleplon in presence of its degradate by HPLC method at 232 nm

Mixture no.	Zaleplon %	Taken ($\mu g\,ml^{-1}$)	Found[a] ($\mu g\,ml^{-1}$)	Found %
1	90	13.50	13.95	100.76
2	80	12.00	12.21	101.75
3	70	10.50	10.69	101.81
4	60	9.00	9.19	102.10
5	50	7.50	7.49	99.87
6	40	6.00	6.12	102.00
7	30	4.50	4.53	100.67
8	20	3.00	3.07	102.30
9	10	2.00	2.04	102.00
Mean ± S.D.				101.47 ± 0.83

[a] Average of three experiments.

times. The assay was performed according to the experimental conditions previously mentioned. The linearity of the calibration graphs and adherence of the system to Beer's law were validated by the high value of the correlation coefficient and the low intercept value (Table 8).

(b) Precision:

Repeatability of the results for concentrations of 5, 7 and 9 $\mu g\,ml^{-1}$ (for both D_2 and ^1DD spectrophotometric methods), 0.3, 0.5 and 0.8 $\mu g\,band^{-1}$ (for spectrodensitometric method) and 2, 7 and 12 $\mu g\,ml^{-1}$ (for HPLC method) were performed by three replicate determinations to estimate intra-day variation (Table 8).

Reproducibility: Seven replicate determinations in different 4 days were used to estimate inter-day variation. The coefficient of variation at these concentration levels was calculated (Table 8).

(c) Range:

The calibration range was established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of Zaleplon present in the pharmaceutical products to give accurate, precise and linear results (Table 8).

Table 7
Application of standard addition technique to the analysis of Zaleplon capsules by HPLC method at 232 nm

Taken ($\mu g\,ml^{-1}$)	Found[a] ($\mu g\,ml^{-1}$)	Found %	Pure added ($\mu g\,ml^{-1}$)	Pure found[b] ($\mu g\,ml^{-1}$)	Recovery %
Siesta® capsules (Batch No. 50507)					
5.00	4.93	98.60	3.00	3.00	100.00
			5.00	5.02	100.40
			10.00	9.91	99.10
			13.00	12.89	99.15
Mean ± S.D.					99.66 ± 0.64
Zalocid® capsules (Batch No. 044060609/3)					
5.00	4.80	96.00	3.00	3.03	101.00
			5.00	5.08	101.60
			10.00	10.10	101
			13.00	13.30	102.31
Mean ± S.D.					101.48 ± 0.62

[a] Average of six experiments.
[b] Average of three experiments.

Table 8
Assay parameters and method validation obtained by applying the proposed methods for determination of Zaleplon

Parameters	Second derivative (D_2) spectrophotometric method	First derivative of ratio spectra (^{1}DD) spectrophotometric method	Spectrodensitometric method	HPLC method
Calibration range	1–10 μg ml^{-1}	1–10 μg ml^{-1}	0.2–1 μg band^{-1}	2–20 μg ml^{-1}
Detection limit (LOD)	0.21 μg ml^{-1}	0.22 μg ml^{-1}	0.05 μg band^{-1}[a]	0.54 μg ml^{-1}
Quantitation limit (LOQ)	0.63 μg ml^{-1}	0.66 μg ml^{-1}	0.2 μg band^{-1}[a]	1.64 μg ml^{-1}
Slope	0.0749	0.2974	0.2174	0.162
Intercept	0.0025	0.0248	0.0504	−0.0032
Mean	100.24	99.9	99.73	100.19
S.D.	0.86	1.07	1.35	1.15
Variance	0.74	1.14	1.83	1.32
Coefficient of variation	0.009	0.01	0.01	0.01
Correlation coefficient	0.9999	0.9999	0.9996	0.9998
R.S.D.%[b]	1.61–0.77–0.74	0.62–0.26–0.64	0.88–1.69–0.92	2.00–1.25–0.82
R.S.D.%[c]	1.48–1.48–1.45	0.77–1.00–0.82	1.02–1.64–1.93	1.63–1.63–0.82

[a] Limit of detection and limit of quantitation for the spectrodensitometric method are determined experimentally.
[b] R.S.D.%: the intra-day relative standard deviation of concentrations (5, 7, and 9 μg ml^{-1}) for D_2 and ^{1}DD spectrophotometric (0.3, 0.5, and 0.8 μg band^{-1}) for spectrodensitometric and (2, 7, and 12 μg ml^{-1}) for HPLC methods, respectively.
[c] R.S.D.%: the inter-day relative standard deviation of concentrations (5, 7, and 9 μg ml^{-1}) for D_2 and ^{1}DD spectrophotometric (0.3, 0.5, and 0.8 μg band^{-1}) for spectrodensitometric and (2, 7, and 12 μg ml^{-1}) for HPLC methods, respectively.

(d) Detection and quantification limits:

According to the ICH recommendations [21], the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits (Table 8), that used for HPLC, D_2 and ^{1}DD spectrophotometric methods.

$$\mathrm{LOD} = 3.3 \times \frac{\mathrm{S.D.}}{\mathrm{Slope}}, \qquad \mathrm{LOQ} = 10 \times \frac{\mathrm{S.D.}}{\mathrm{Slope}}$$

N.B.: For the spectrodensitometric method, both are determined experimentally.

- Experimentally the detection limit is defined as the concentration of the analyte producing a signal which is at least three times the base line noise measured from peak to peak. The quantitation limit is defined as the concentration of the analyte producing the signal which is at least ten times the base line noise.

(e) Selectivity:

Selectivity of the methods was achieved by the analysis of different laboratory prepared mixtures of Zaleplon and its degradate within the linearity range. Satisfactory results were shown (Tables 1, 4 and 6).

(f) Accuracy:

Accuracy of the methods was assured by the use of the standard addition technique; it was performed by addition of known amounts of pure Zaleplon to a known concentration of the commercial capsules. The resulting mixtures were assayed and the results obtained for Zaleplon were compared with the expected results. The good recoveries of standard addition technique (Tables 3, 5 and 7), suggested good accuracy of the proposed methods.

(g) Stability:

Zaleplon working solutions showed no spectrophotometric, and chromatographic changes for 3 days when stored refrigerated at 5 °C (stability indicating method).

Table 9
Statistical analysis of parameters required for system suitability testing of HPLC method

Parameters	Obtained value	Reference value
Resolution (R)	3.9	$R > 0.8$
Relative retention (α)	2.2	>1
Tailing factor (T)	1.24	<1.5–2 or <2[a]
Capacity factor (K')	2.4	1–10 acceptable
Column efficiency (N)	1648	Increase with efficiency of the separation
HETP[b]	$10^{-2} \times 1.33$	The smaller the value the higher the column efficiency

[a] Refers to ref. [22].
[b] Height equivalent to theoretical plate (cm plate^{-1}).

(h) Robustness:

Different mobile phases contain water, acetonitrile, methyl alcohol with different ratios and pH using phosphate buffer were tried to obtain the optimum parameters for complete separation.

(i) System suitability testing for HPLC:

System suitability test of HPLC method gave good resolution ($R = 3.9$), relative retention time ($\alpha = 2.2$), column capacity ($K = 2.4$), and tailing factor ($T = 1.24$) (Table 9).

6. Conclusion

It is clear from the degradation pathway of Zaleplon (Fig. 1) that it is highly susceptible to hydrolysis. The proposed methods are efficient for providing simple, accurate and reproducible quantitative analysis for the determination of Zaleplon in pharmaceutical tablets, without any interference from excipients or its degradation product.

D_2 and ^{1}DD spectrophotometric methods have the advantages of low cost and speed. On the other hand, the spectrodensitometric and HPLC methods were found to be

Table 10
Statistical comparison of the results obtained by the four proposed methods and the reference method

Parameters	Second derivative (D_2)		First derivative of ratio spectra (^{1}DD)		Spectrodensitometric method		HPLC method		Reference method[a]	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Mean%	97.2	96.0	97.40	96.60	98.00	97.00	98.60	96.00	97.18	95.01
S.D.	1.04	1.04	1.00	1.06	1.19	1.28	0.94	0.73	1.09	1.37
N	6	6	6	6	6	6	6	6	6	6
Variance	1.07	1.07	1.00	1.13	1.41	1.63	0.88	0.53	1.20	1.89
Student's *t*-test (2.23)[b]	0.97	0.17	0.72	0.06	0.24	0.03	0.04	0.14		
F-test (5.05)[b]	1.12	1.77	1.19	1.67	1.18	1.16	1.35	3.55		

N.B.—*a*: Siesta® capsules (Batch No. 50507) and *b*: Zalocid® capsules (Batch No. 044060609/3).
[a] Reference method is the company method.
[b] Figure between parentheses represent the corresponding tabulated values of *t* and *F* at $P = 0.05$.

highly selective but spectrodensitometric method was found to be the most sensitive.

The proposed techniques are more selective than the company method which is RP-HPLC using acetonitrile:water:acetic anhydride (70:29.5:0.5, v/v/v) with UV detection at 280 nm using hypersil-ODS C_{18} column and flow rate 1 ml min^{-1}. Applying the company method to the drug and its degradate, it was found that they cannot be separated from each other, and cannot be used for Zaleplon analysis in presence of its degradate. In addition, the proposed HPLC method has an advantage of using an internal standard.

The results of the proposed methods were statistically compared with the company method [g2] on the capsules. The *t* and *f* values were computed (by Microsoft Excel Program) and found to be less than the tabulated ones indicating no significant difference with respect to accuracy and precision (Table 10).

Furthermore, statistical analysis of the results obtained by the proposed methods and the company method were performed with Microsoft Excel Program using one-way analysis of variance (ANOVA; *F*-test) at $P < 0.05$. The test ascertained that the proposed methods are as precise and accurate as the company method (Table 11).

The results obtained indicate that the proposed methods are rapid, simple and sensitive stability indicating procedures. Economically, all the analytical reagents are inexpensive, have excellent shelf life and available in any analytical laboratory. These merits suggest the use of the proposed methods in routine and quality control analysis without interference of commonly encountered addivities.

Table 11
Statistical analysis of the results obtained by applying the four proposed methods and the reference method for Siesta® and Zalocid® tablets using one-way ANOVA (*F*-test)

Method	Mean ± R.S.D.%[a]	
	a	*b*
Second derivative (D_2)	97.20 ± 1.07	96.00 ± 1.08
First derivative of ratio spectra (^{1}DD)	97.40 ± 1.03	96.60 ± 1.10
Spectrodensitometric method	98.00 ± 1.21	97.00 ± 1.32
HPLC method	98.60 ± 0.95	96.00 ± 0.76
F value[b]	1.9	2.62

a: Siesta® capsules (Batch No. 50507) and *b*: Zalocid® capsules (Batch No. 044060609/3). $n = 6$.
[a] R.S.D. = relative standard deviation.
[b] There was no significance difference between the methods using one-way ANOVA (*F*-test), where *F* tabulated = 2.76.

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