Journal of Chromatographic Science 2015;53:1395–1399
doi:10.1093/chromsci/bmv015 Advance Access publication March 3, 2015

Article

HPTLC Method for Quantitative Determination of Zopiclone and Its Impurity

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Received 23 August 2014; revised 19 December 2014

This study was designed to establish, optimize and validate a sensitive, selective and accurate high-performance thin layer chromatographic (HPTLC) method for determination of zopiclone (ZPC) and its main impurity, 2-amino-5-chloropyridine, one of its degradation products, in raw material and pharmaceutical formulation. The proposed method was applied for analysis of ZPC and its impurity over the concentration range of 0.3–1.4 and 0.05–0.8 μg/band with accuracy of mean percentage recovery 99.92% ± 1.521 and 99.28% ± 2.296, respectively. The method is based on the separation of two components followed by densitometric measurement of the separated peaks at 305 nm. The separation was carried out on silica gel HPTLC F_{254} plates, using chloroform–methanol–glacial acetic acid (9:1:0.1, by volume) as a developing system. The suggested method was validated according to International Conference on Harmonization guidelines and can be applied for routine analysis in quality control laboratories. The results obtained by the proposed method were statistically compared with the reported method revealing high accuracy and good precision.

Introduction

Zopiclone (ZPC), Figure 1, chemically known as 6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5*H*-pyrrolo(3,4-b)pyrazin-5-yl-4-methylpiperazine-1-carboxylate (1), is a cyclopyrrolone compound with anxiolytic, muscle relaxant, sedative, amnestic and anticonvulsant properties like the benzodiazepines. Its actions are mediated by activation of gamma-amino butyric acid (GABA) in the brain. ZPC is used as a hypnotic in the short-term treatment of insomnia (2).

ZPC is assayed in the British Pharmacopeia (BP) as an official compound by the non-aqueous titrimetric method with potentiometric detection of end point for its assay in pure form, and the HPLC method for assay in tablets (3). The literature survey reports several analytical methods for the determination of ZPC, which include spectrophotometry (4, 5), ion-selective electrode (6), polarography (6, 7), voltammetry (8), thin layer chromatography (TLC) (9), high-performance liquid chromatography (HPLC) (4, 10–15) and gas chromatography (GC) (14, 16, 17).

2-Amino-5-chloropyridine (ACP) (Figure 1) was defined by BP (3) as a potential impurity in ZPC tablets; as such it specifies that its limit should not exceed 0.5%. Some studies were reported the instability of ZPC as a cyclopyrrolone compound (13, 18, 19). ACP was identified to be the alkaline degradation product of ZPC. Ring opening of the pyrrolidone ring yields an intermediate product that is hydrolyzed to ACP (19). Furthermore, ACP was identified as the degradation product of ZPC in stability tests in human blood samples (20).

It is reported that ACP is harmful if swallowed, inhaled or absorbed through the skin. It may cause irritation of respiratory tract, skin and eyes (21).

There was a strong need to develop a new simple, sensitive and accurate stability-indicating assay method that can detect and quantitate ZPC in raw material and pharmaceutical formulation, besides offering detection and quantitation of its main impurity/degradation product without need of expensive complicated methods.

The aim of this study is to establish, optimize and validate accurate, sensitive and selective HPTLC method for the determination of ZPC and its impurity/degradation product in raw material and pharmaceutical formulation.

Experimental

Instruments

These include Camag TLC Scanner 3 plus Camag TLC sampler Linomat IV (Camag, Muttenz, Switzerland) supplied with a 100 μL syringe for HPTLC-densitometric determinations.

The following requirements are taken into consideration: slit dimensions: 6 × 0.3 mm; scanning speed: 20 mm/s; spraying rate: 10 μL/s; data resolution: 100 μm/step; band width: 6 mm; result output: chromatogram and integrated peak area. HPTLC aluminum plates (20 × 20 cm) were coated with 0.25 mm silica gel 60 F_{254} (Merck, Germany).

Material and reagents

Pure standard

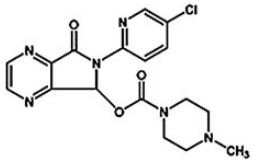
ZPC pure sample was kindly provided by Amoun Pharmaceutical Co., Cairo, Egypt. Its purity was found to be 99.58% based on the company analysis certificate. ACP was purchased from Sigma–Aldrich Co. (Germany) with a purity of 98%.

Pharmaceutical formulation

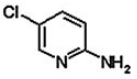
Hypnor® tablets manufactured by Amoun Pharmaceutical Co.; batch no. 121084 were labeled to contain 7.5 mg ZPC/tablet and were purchased from the local market.

Chemicals and reagents

All chemicals and solvents used were of analytical grade, which include methanol HPLC grade (E. Merck, Germany) and chloroform and glacial acetic acid (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).



Zopiclone (ZPC)
$C_{17}H_{17}ClN_6O_3$
Mol. Wt. 388.81



2-amino-5-chloropyridine (ACP)
$C_5H_5ClN_2$
Mol. Wt. 128.56

Figure 1. Chemical structure of ZPC and ACP.

Standard solutions

These include stock solutions of ZPC and ACP (1 mg/mL)—0.05 g of each of ZPC and ACP were accurately transferred into two separate 50-mL volumetric flasks, dissolved in and diluted to volume with methanol—and working solutions of ZPC and ACP (100 μg/mL)—5 mL of ZPC and ACP stock solutions were accurately transferred into two separate 50-mL volumetric flasks and completed to volume with methanol.

Procedure

Linearity and construction of calibration curve

Aliquots equivalent to 0.3–1.4 mg of ZPC and 0.05–0.8 mg of ACP were transferred separately from their stock standard solutions (1 mg mL^{-1}) into a series of 10-mL volumetric flasks, and then the volume was completed with methanol. Then, 10 μL of each solution of ZPC and ACP were applied in triplicate to HPTLC plates (20 × 10 cm) as bands with 6 mm width using a Camag Linomat IV applicator. The bands were spaced 5 mm from each other and 10 mm apart from the bottom edge of the plate. Linear ascending development was performed in a chromatographic chamber previously saturated with chloroform–methanol–glacial acetic acid (9:1:0.1, by volume) as a developing system for half hour at room temperature to a distance of 9 cm. The integrated peak areas were recorded using scanning wavelength at 305 nm under the specified instrumental conditions. The calibration curves of ZPC and ACP were constructed by plotting the mean integrated peak area/10,000 versus the corresponding concentration and then the regression equations were computed.

Assay of pharmaceutical formulation (Hypnor® tablets)

Twenty Hypnor® tablets were weighed and crushed to obtain a fine powder. An accurately weighed portion equivalent to 100 mg of ZPC was transferred into a 100-mL volumetric flask and then 75 mL methanol was added. The prepared solution was sonicated for 30 min, cooled and completed to volume with methanol. The solution was filtered and diluted to obtain 100 μg/mL working solution. The procedure detailed under "Linearity and construction of calibration curve" section was followed and then ZPC concentration was calculated using the corresponding regression equation. When carrying out the standard addition technique, the tablet powder and pure ZPC were mixed well together before proceeding in the above-mentioned procedures.

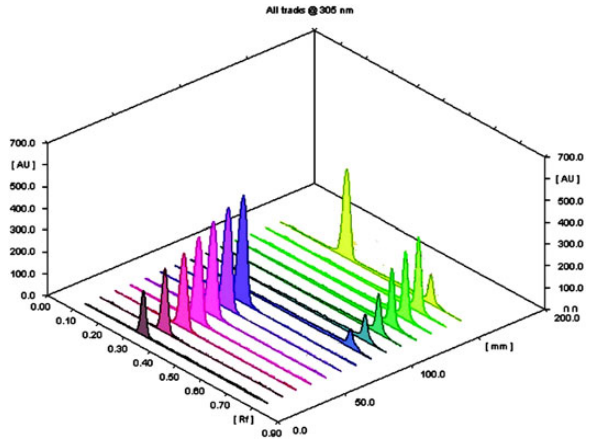


Figure 2. HPTLC densitogram of ZPC ($R_f = 0.36 \pm 0.01$) in the concentration range (0.3–1.4 μg/band), ACP ($R_f = 0.72 \pm 0.01$) in the concentration range (0.05–0.8 μg/band) and a mixture of ZPC and ACP using (chloroform–methanol–glacial acetic acid, 9:1:0.1, by volume) as a developing system at 305 nm. This figure is available in black and white in print and in color at *JCS* online.

Results

It was necessary to study the effect of different parameters to obtain maximum resolution.

Developing system

Different developing systems of different compositions and ratios were tried, which include chloroform–methanol (9:1, by volume), chloroform–methanol–ammonia solution (8:2:0.2, by volume), chloroform–methanol–glacial acetic acid (8:2:0.2, by volume) and chloroform–acetone–triethylamine (8:2:0.2, by volume), to obtain optimum separation between ZPC and its degradation product (ACP). The best developing system was found to be chloroform–methanol–glacial acetic acid (9:1:0.1, by volume) (Figures 2 and 3).

Scanning wavelength

Different scanning wavelengths were tried (254, 243 and 305 nm) in order to obtain good sensitivity of ZPC and ACP with minimum noise. The wavelength 305 nm was found to be the best wavelength regarding sensitivity of ZPC and its impurity ACP. Peaks were sharp and symmetrical with minimum noise, as shown in Figures 2 and 3.

Slit dimensions of scanning light beam

The slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of the adjacent band. Different slit dimensions were tried and 6 × 0.3 mm was proved to be the slit dimensions of choice that provided highest sensitivity.

ZPC was successfully separated from its impurity ACP using TLC. The method was applied for the determination of ACP in ZPC pure substance and tablets in trace concentrations, so it can be applied to detect very low extent of impurity of ZPC (Table I). This method offers high sensitivity and selectivity for analysis of ZPC and ACP using chloroform–methanol–glacial acetic acid (9:1:0.1, by volume) as mobile phase, where the good separation is shown by the difference in the retention

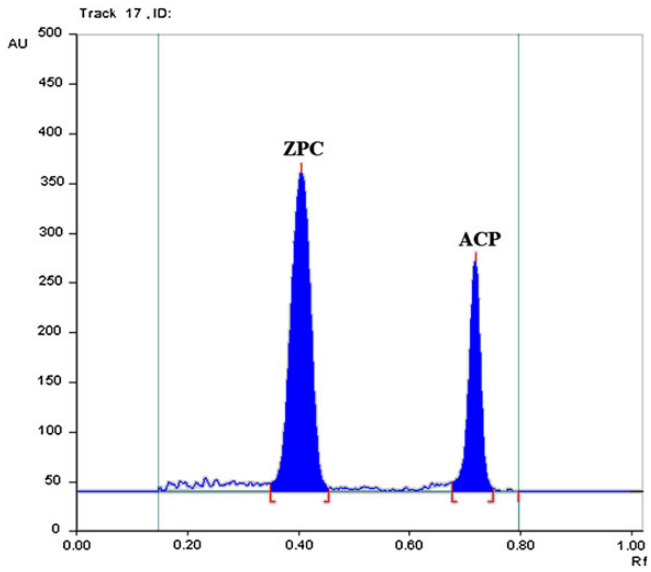


Figure 3. 2D HPTLC densitogram of separated peaks of ZPC ($R_f = 0.36 \pm 0.01$) and ACP ($R_f = 0.72 \pm 0.01$) using (chloroform–methanol–glacial acetic acid; 9:1:0.1, by volume) as a developing system at 305 nm. This figure is available in black and white in print and in color at *JCS* online.

Table I
Regression and Analytical Parameters of the Proposed Method for Determination of ZPC and ACP

Parameter	HPTLC	
	ZPC	ACP
Calibration range	0.3–1.4 μg/band	0.05–0.8 μg/band
Slope	0.9428	0.8546
Intercept	0.3207	0.1930
Correlation coefficient (*r*)	0.9998	0.9999
Accuracy	99.92% ± 1.521	99.28% ± 2.296
Precision: repeatability (RSD%)a[a]	1.16	1.58
Intermediate precision (RSD%)b[a]	2.50	2.86
Robustness parameters (RSD%)		
Chloroform (9 ± 0.5 mL)	0.167	0.082
Glacial acetic acid (0.1 ± 0.05 mL)	0.097	0.046
LOD[b]	0.02 μg/band	0.01 μg/band
LOQ[b]	0.30 μg/band	0.05 μg/band

[a](RSD%)a and (RSD%)b; the intra- and inter-day RSD of concentrations (0.4, 0.8 and 1.2 μg/band) for ZPC and (0.1, 0.2 and 0.5 μg/band) for ACP.
[b]Limit of detection and quantitation are determined experimentally by the signal-to-noise ratio.

factor (R_f) values of ZPC ($R_f = 0.36 \pm 0.01$) and ACP ($R_f = 0.72 \pm 0.01$) (Figures 2 and 3).

Linear correlations were obtained between the peak area/10,000 (Y) and the corresponding concentrations (X in μg/band; Table I), where the regression equations were calculated as

$$Y = 0.9404X + 0.3235, \quad r = 0:9998 \text{ for ZPC};$$
$$Y = 0.8546X + 0.1930, \quad r = 0.9999 \text{ for ACP}.$$

The calibration curve was constructed with mean percentage recoveries 99.92% ± 1.521 and 99.28% ± 2.296 for ZPC and ACP, respectively.

The proposed methods have been applied to assay ZPC in Hypnor® tablets. The validity of the method was further assessed by applying the standard addition technique (Table II). The results obtained indicate no interference from dosage form additives present with the studied mixture.

Table II
Determination of ZPC in Pharmaceutical Formulation by the Proposed Method and Application of the Standard Addition Technique

Pharmaceutical formulation	HPTLC-densitometric method			
	Taken (μg/band)	Found %[a] ± SD	Pure added (μg/band)	Recovery, %[b]
Hypnor® tablets B.N. 121084	0.3	98.47 ± 1.816	0.2	102.41
			0.3	98.04
			0.4	97.65
		Mean ± SD		99.37 ± 2.643

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

Discussion

The HPTLC method has advantages for routine quantitative determination of being selective, sensitive, accurate and rapid analytical method and reduces sample preparations, laboratories consumption and cost materials. It has advantages over the HPLC method of being cost-effective and time saving in analysis. This method is used frequently as stability indicating method for determination of many drugs in the presence of their degradation products or impurities (22–24).

The method allows determination of ZPC in the presence of its impurity ACP which is also its degradation product (13, 19, 20) using chloroform–methanol–glacial acetic acid (9:1:0.1, by volume) as mobile phase, where the good separation is shown by the difference in the retention factor (R_f) values of ZPC ($R_f = 0.36 \pm 0.01$) and ACP ($R_f = 0.72 \pm 0.01$) (Figures 2 and 3).

The linearity of the proposed method was evaluated by analyzing different concentrations of ZPC and ACP. Good linearity is expressed by the high value of the correlation coefficient.

It is used to determine ACP in ZPC pure substance in trace concentrations down to 0.05 μg/band. It can be applied as stability-indicating assay method of ZPC, in addition to purity testing of ZPC tablets. Furthermore, the suggested procedures were applied for the routine quality control of ZPC commercial tablets.

Table II shows that the suggested method is valid and applicable for the analysis of ZPC in Hypnor® tablets with an acceptable percentage recovery. Moreover, the validity of the proposed method was assessed by applying the standard addition technique, which showed accurate results and there was no interference from tablet excipients.

The results obtained by the proposed method were statistically compared with those obtained by the reported method using *t*- and *F*-tests. The values obtained are less than the theoretical values indicating that there is no significant difference between the proposed methods and the reported spectrophotometric method (4) with respect to accuracy and precision (Table III).

Methods validation

Methods validation was performed according to the International Conference on Harmonization (ICH) guidelines (25) for the proposed method.

Linearity

Under optimum experimental conditions, ZPC was determined in triplicates in the range of 0.3–1.4 μg/band. ACP was also

Table III
Statistical Analysis of the Proposed HPTLC Method and the Reported Spectrophotometric Method for Determination of ZPC in Hypnor® tablets

Parameters	HPTLC	Reported method[a]
Mean	98.47	98.55
SD	1.816	1.364
Variance	3.301	1.856
N	6	6
Student's *t*-test[b] (2.228)	0.087	–
F-test[b] (5.050)	1.778	–

[a]UV-spectrophotometric methods for the determination of ZPC in tablets at 304 nm using acetonitrile as a solvent (4).
[b]The values between parenthesis are corresponding to the theoretical values of *t* and *F* ($P = 0.05$).

determined in triplicates in the range of 0.05–0.8 μg/band. 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 intercept value (Table I).

Range

The specified range is derived from linearity studies and depends on the application of analytical procedure. The concentration of ZPC present in pharmaceutical preparation gave accurate, precise and linear results with the suggested method (Table I).

Accuracy

Accuracy was assessed by the standard addition technique and through analysis of market pharmaceutical preparation by the proposed method. The resulting synthetic mixtures were assayed and the results obtained were compared with those expected. The good recoveries of pure drug samples suggest good accuracy of the proposed methods (Table I).

Precision

Repeatability

Three concentrations of both ZPC (0.4, 0.8 and 1.2 μg/band) and ACP (0.1, 0.2 and 0.5 μg/band) of ACP were determined in triplicates in the same day to estimate intraday variation. Good results and acceptable relative standard deviation (RSD %) are shown in Table I.

Intermediate precision

The previous procedures were repeated on the same concentrations in triplicate on three successive days to determine the intermediate precision. Good results and acceptable % RSD are shown in Table I.

Specificity

Specificity of the HPTLC method is expressed through the good separation of the two components as shown in Figures 2 and 3.

Detection and quantitation limits

Both are determined by the signal-to-noise ratio. The signal-to-noise ratio is determined by comparing the measured signals of samples with known low concentrations of analytes with those of blank samples. The detection limit is defined as the concentration of the analyte producing a signal which is at least three times the baseline 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 baseline noise (25). Acceptable detection and quantitation limits are shown in Table I.

Table IV
Parameters of System Suitability of the Developed HPTLC Method for the Determination of ZPC and ACP

Parameters	ZPC	ACP	Reference value (25)
Capacity factor (k')	1.78	0.39	0–10
Symmetry factor	0.99	0.98	~1
Resolution (Rs)	5.23		$R > 2$
Selectivity (α)	2.00		$\alpha > 1$

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small deliberate variations in method parameters and provides an indication of its reliability during normal usage (25). Changing the organic strength of the developing system by ± 0.5 mL has no significant effect on R_f values or area under the peaks. A change in volume of glacial acetic acid by ± 0.05 mL has been also studied showing an effect on R_f values of the bands, although good separation still exists. The % RSD was calculated, and the results are given in Table I.

System suitability

ICH (25) states that system suitability tests are an integral part of many analytical methods, especially liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Parameters including capacity factor (k') (26), symmetry factor, resolution (Rs) and selectivity factor (α) were calculated as shown in Table IV.

Conclusion

The proposed method represents the sensitive, accurate and rapid stability-indicating assay method for ZPC, which has advantages over the previously published methods. The method is a sensitive one allowing the determination of ACP in ZPC. The HPTLC method was applicable for assay and purity testing of ZPC in bulk and pharmaceutical formulations without interference of additives in the pharmaceutical preparation.

The advantages of the HPTLC method are that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering analysis time and cost per analysis and provides high sensitivity and selectivity, besides advantages of HPTLC over TLC-densitometry of using plates with smaller particle size and higher resolution ability. The results obtained indicate that the introduced method can be classified among the highly selective and sensitive procedures.

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