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Simultaneous determination of zopiclone and its degradation product and main impurity (2-amino-5-chloropyridine) by micellar liquid chromatography with time-programmed fluorescence detection: Preliminary investigation for biological monitoring

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ABSTRACT

A simple and reliable HPLC method was developed and validated for the simultaneous determination of the hypnotic drug, zopiclone (ZPC) and its degradation product and main impurity, 2-amino-5-chloropyridine (ACP). The analyses were carried out on BDS Hypersil phenyl column (4.6 mm × 250 mm, 5 μm particle size) using micellar mobile phase consisting of 0.15 M SDS, 10% n-propanol, 0.3% triethylamine, and 0.02 M orthophosphoric acid (pH 3.5) with timed programmable fluorescence detection. The proposed method was found to be rectilinear over the concentration ranges of 0.5–10.0 μg/mL for ZPC and 2.5–50 ng/mL for ACP. Moreover, the method was applied for the determination of ZPC in commercial tablets with mean percentage recovery of 99.06 ± 1.49. The results of the proposed method were statistically compared with those obtained by the comparison method revealing no significance differences in the performance of the two methods regarding accuracy and precision. Furthermore, the proposed method was applied for the detection and determination of ACP in human urine as a marker for ZPC intake.

1. Introduction

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

Zopiclone is an official compound in the British pharmacopeia (BP) [2] that described a non-aqueous titrimetric method with potentiometric detection of end point for its determination in pure form and an HPLC method for its determination in tablets. Literature review revealed many analytical methods for the determination of ZPC including spectrophotometry [3], HPLC [3–9], GC [9–12], TLC [13], ion-selective electrode [14] and polarographic methods [14,15].

It is known that the cyclopyrrolone compounds are quite unstable; the instability of ZPC was the subject of some publications [8,16–18]. Fernandez et al. [16] studied the degradation of ZPC in different buffered media. Mannaert [17] identified the degradation product of ZPC to be 2-amino-5-chloropyridine (ACP, Fig. 1). Also an HPLC method [8] was applied as stability-indicating assay method for ZPC. Moreover, the BP [2] defines 2-amino-5-chloropyridine (ACP) as a potential impurity in ZPC tablets; as such it specifies its limit not to exceed 0.5%.

Degradation of ZPC occurs through ring opening of the pyrrolidone ring yielding intermediate structure that is hydrolyzed to ACP (Fig. 1, 18). Toxicological properties of ACP have not been thoroughly investigated, but it is reported to be harmful if swallowed, inhaled or absorbed through the skin. It may cause irritation of respiratory tract, skin and eyes [19]. The instability phenomenon of ZPC was exploited as a tool for the detection of ZPC in a general drug screening [18].

A comprehensive literature search revealed that many of the reported methods failed to separate the parent drug from its degradation product and thus lack the stability-indicating nature [3–7,9–15]. On the other hand, few methods were applied as stability-indicating assays for ZPC, those methods are either not sufficiently sensitive to detect trace amounts of ACP in ZPC drug

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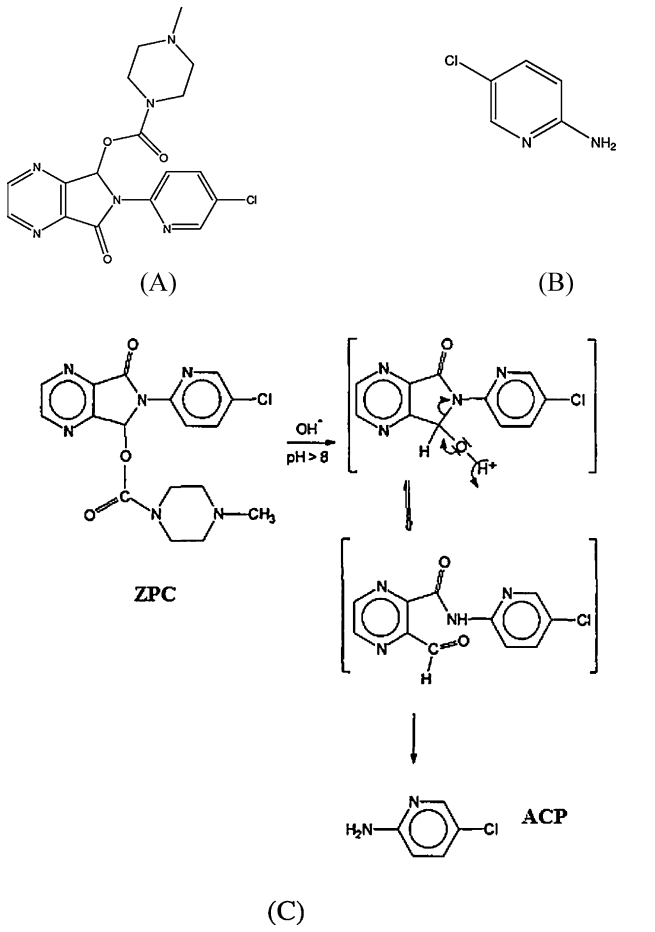

Fig. 1. (A) Structural formula of zopiclone. (B) Structural formulae of 2-amino-5-chloropyridine. (C) Degradation pathway of zopiclone to 2-amino-5-chloropyridine [18].

substance [16–18], or even they are time consuming requiring long run time to separate the drug from its degradation product [8]. Considering biological applications, the reported methods for determination of ZPC in biological fluids require elaborate extraction steps through traditional liquid–liquid extraction procedures with a considerable amount of toxic and flammable organic solvents for sample preparation [4,5,9], or solid phase extraction procedures [7,10,18] which requires multiple steps and special cartridge. Therefore, there was a strong need to develop a new stability-indicating assay method that can detect and quantitate ACP in ZPC drug substance and pharmaceutical preparations, and also can be applied to biologically monitor ZPC intake rapidly without the need for sample pretreatment or multiple extraction steps.

Micellar liquid chromatography (MLC) is a mode of reversed phase liquid chromatography, which uses aqueous solutions of surfactants above the critical micellar concentration. This chromatographic system presents some differences with respect to the classical reversed phase liquid chromatography, because the stationary phase is modified by the absorption of surfactant and the mobile phase presents surfactant micelles. This system provides hydrophobic, electronic and steric sites of interaction for solutes that allows the effective separation of compounds of different natures [20,21]. MLC allows biological samples to be analyzed without prior protein precipitation and eliminating other interfering substances, thus considerably reducing the cost and analysis time. In addition, the use of micellar mobile phases reduces the consumption of organic solvents and thus meets the requirement of "green chemistry" concept [20,21].

In our laboratory, MLC proved to be a useful technique in the quality control of several drugs in pharmaceutical preparations and biological fluids using hybrid micellar mobile phase containing SDS and an organic modifier [22–24].

The aim of the present study is to develop and validate a sensitive and rapid stability-indicating micellar liquid chromatographic method for determination of ZPC in raw material and tablets. The proposed MLC method provided a sensitive tool that allowed ACP to be rapidly identified and quickly determined in ZPC pure form and tablets even if it exists in a very low concentration down to 0.025%. The proposed method was extended to quality control analysis of ZPC tablets. The applicability of the proposed MLC method for the detection of ACP in urine samples was examined. The proposed MLC method provided a simple and rapid procedure for the screening of ACP in urine without any pretreatment or extraction steps except sample dilution with the mobile phase.

2. Experimental

2.1. Apparatus

2.1.1. HPLC system

Separations were performed using a Shimadzu LC-20AD chromatograph (Japan) equipped with a Rheodyne injector valve with a 20 μL loop and a RF-10AXL fluorescence detector. Mobile phases were degassed using DGU-20A5 solvent degasser. CBM-20A Communication Bus Module was used to connect the instrument to PC computer.

2.1.2. pH-meter

A Consort P901 pH meter (Belgium) was used for pH-adjustment.

2.2. Materials and reagents

All solvents used were of HPLC grade, all the chemicals were of analytical reagent grade and distilled water was used throughout the study.

- Zopiclone pure sample (certified purity of 99.0%) was kindly provided by Amoun Pharmaceutical Co., Cairo, Egypt.
- 2-Amino-5-chloropyridine was purchased from Sigma–Aldrich Co. (Germany).
- Hypnor® tablets labeled to contain 7.5 mg zopiclone/tablet (product of Amoun Co., Cairo, Egypt) were purchased from a local pharmacy.
- Acetonitrile, methanol, n-propanol (HPLC grade) and sodium dodecyl sulfate (SDS, 99%) were purchased from Sigma–Aldrich Co. (Germany).
- Triethylamine (TEA) and orthophosphoric acid (85%, w/v) were obtained from Riedel-deHäen (Sleeze, Germany).
- Cellulose acetate syringe filters with 0.45 μm pore size (Gemma Medical, Barcelona, Spain).
- Urine samples were freshly obtained from a healthy 40 years old female volunteer.

2.3. Chromatographic conditions

Column: BDS Hypersil phenyl column (4.6 mm × 250 mm, 5 μm particle size), Thermo Electron Corporation, Runcorn, UK.
Mobile phase: A micellar mobile phase consisting of 0.15 M SDS, 10% n-propanol and 0.3% TEA was prepared in 0.02 M orthophosphoric acid. The pH of the mobile phase was adjusted to pH 3.5. The mobile phase was filtered through 0.45 μm Millipore membrane filter and degassed by sonication for 30 min before use.

Flow rate: 1 mL/min.
Fluorescence detection program: 0–5.6 min at 305/370 nm and after 5.6 min at 320/480 nm.

2.4. Preparation of stock and standard solutions

Stock solutions containing 100.0 μg/mL of each of ZPC and ACP were prepared in acetonitrile. Standard solutions containing 10.0 μg/mL of ACP were freshly prepared daily by appropriate dilution of the stock solution with acetonitrile. The stock solutions were found to be stable for one week when kept in the refrigerator at 4 °C.

2.5. General recommended procedures

2.5.1. Construction of calibration graphs

Working solutions containing 0.5–10.0 μg/mL of ZPC or 2.5–50.0 ng/mL of ACP were prepared by serial dilution of the standard solutions with the mobile phase. 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The average peak areas of ZPC and ACP were plotted *versus* the corresponding drug concentrations and the corresponding regression equations were derived.

2.5.2. Analysis of laboratory-prepared mixtures of ZPC and ACP

Aliquots of ZPC with ACP standard solutions in different ratios were transferred into a series of 10 mL volumetric flasks. The volume was completed to the mark with the mobile phase and mixed well. 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The percentage recoveries of ZPC were determined either from the previously constructed calibration graph or from the corresponding regression equation.

2.5.3. Analysis of tablets

Ten tablets were accurately weighed, finely pulverized and thoroughly mixed. An accurately weighed amount of the powder corresponding to 5.0 mg of ZPC declared active principle was transferred into 50 mL volumetric flask. About 40 mL of acetonitrile was added and the mixture was sonicated in an ultrasonic bath for 30 min. The volume was completed to the mark with the same solvent. Excipients were not soluble in this medium and, hence, the solution had to be filtered through 0.45 μm cellulose acetate syringe filter before injection into the chromatograph. Accurately measured aliquots of the filtrate were transferred into a series of 10 mL volumetric flasks, diluted to the mark with the mobile phase and mixed well to obtain final ZPC concentrations of 5.0, 8.0, and 10.0 μg/mL. 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The nominal contents of the tablets were calculated either from the calibration graph or from the corresponding regression equation.

2.5.4. Construction of calibration graph for ACP in urine

1 mL aliquots of human urine sample were transferred into a series of 10 mL volumetric flasks and spiked with increasing concentrations of ACP (5.0–50.0 ng/mL). The volume was completed to the mark with the mobile phase and the contents of the flasks were mixed well. The solutions were filtered through 0.45 μm cellulose acetate syringe filter then 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The average peak areas of ACP were plotted *versus* the corresponding concentrations to get the calibration graph and the corresponding regression equation was derived.

2.5.5. Application of the proposed method for the analysis of ACP in spiked urine samples

1 mL aliquots of human urine sample were transferred into 10 mL volumetric flasks and spiked with ACP over the concentration range of 5.0–50.0 ng/mL. The volume was completed to the mark with the mobile phase and the contents of the flasks were mixed well. The solutions were filtered through 0.45 μm cellulose acetate syringe filter then 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The concentration of ACP was calculated from the regression equation.

3. Results and discussion

The proposed MLC method permitted the separation of ZPC from its degradation product, ACP. The method allowed the determination of ACP in ZPC pure substance and tablets in trace concentrations down to 0.025%, so it can be applied to detect very low extent of degradation of ZPC, in addition to purity testing of ZPC tablets within the pharmacopeial limit stated by the BP which is 0.5% of ACP [2]. Furthermore, the suggested procedure was applied for the quality control of ZPC commercial tablets.

By virtue of sensitivity of the proposed method for determination of ACP (low ng level), it was applied to determine ACP in human urine to detect the intake of the hypnotic drug, ZPC. The use of a micellar mobile phase allows the direct injection of urine samples without the need for extraction steps, only dilution of the sample with the micellar mobile phase is sufficient to dissolve sample proteins and other endogenous compounds.

3.1. Selection and optimization of chromatographic conditions

To achieve the best chromatographic conditions, the mobile phase composition was optimized to provide sufficient selectivity and sensitivity in a short separation time. The different chromatographic conditions affecting the separation and resolution of ZPC and ACP were carefully studied and optimized. The results of the optimization study are summarized in Table 1.

3.1.1. Choice of column

Two columns were tested for performance investigation, these include:

1. CLC Shim-pack C_{18} column (250 mm × 4.6 mm, 5 μm particle size), Shimadzu Corporation, Japan.
2. BDS Hypersil phenyl column (250 mm × 4.6 mm, 5 μm particle size), Thermo Electron Corporation, Runcorn, UK.

Using C_{18} column resulted in very long retention times of the drug (more than 20 min) and its degradation product (10 min). Consequently, more polar column was tried to decrease the retention of the drug and its degradation product. A phenyl column was found to be appropriate since it gave symmetrical well resolved peaks within a reasonable analytical run time (about 7 min).

3.1.2. Choice of appropriate detection wavelength and time program

The fluorescence detector was found to be superior to UV detector to develop a stability-indicating assay for ZPC since its degradation product (ACP) exhibits a very strong fluorescence allowing very sensitive detection. The optimum excitation and emission wavelengths were determined *via* online fluorescence scanning of ZPC and ACP. ZPC was found to exhibit maximum fluorescence intensity at 480 nm after excitation at 320 nm, on the other hand, ACP was found to exhibit an intense native fluorescence at 370 nm after excitation at 305 nm. Programmable fluorescence

Table 1
Optimization of chromatographic conditions for separation of ZPC and ACP.

Parameter	No. of theoretical plates (N)		Capacity factor (k')		Tailing factor (T)		Resolution (R_s)	Relative retention (α)
	ZPC	ACP	ZPC	ACP	ZPC	ACP		
pH								
3.5	1560	2170	1.92	0.81	1.2	1.4	4.2	1.23
4.5	1260	2060	1.63	0.99	1.2	1.5	2.3	1.26
5.5	1100	2000	1.66	0.94	1.5	2.0	2.8	1.37
6.5	1000	2100	1.24	0.74	1.4	1.7	2.2	1.23
Conc. of SDS (M)								
0.05	1870	4040	7.95	2.23	1.1	1.1	11.1	2.77
0.10	1170	3070	2.90	1.04	1.1	1.3	6.2	1.91
0.12	1090	2850	2.44	0.94	1.1	1.2	5.3	1.78
0.15	1000	2100	1.92	0.74	1.4	1.7	4.2	1.67
Conc. of n-propanol (%, v/v)								
8	1150	2478	2.21	1.67	1.1	1.5	1.8	1.21
10	1000	2100	1.92	0.74	1.4	1.7	4.2	1.23
12	1310	1680	1.33	0.83	1.2	1.5	2.3	1.27
15	1580	1660	1.07	0.64	1.2	1.5	2.3	1.26

detection was employed to allow sensitive determination of both ZPC and ACP simultaneously. ACP was detected at 305/370 within 0–5.6 min, while, ZPC was detected at 320/480 nm after 5.6 min.

3.1.3. Effect of pH of the mobile phase

The pH is the first parameter to be studied when a MLC procedure is going to be performed. Both ZPC and ACP are basic compounds, their pK_a values are 6.7 and 9.3, respectively. Consequently, the working pH range (3.5–6.5) is not an influential parameter on the retention of the two compounds since these substances are protonated in this range. ZPC and ACP were eluted with mobile phases of pH values over the range of 3–6.5, obtaining the same retention times (≈4.9 and 6.4 min for ACP and ZPC, respectively). pH 3.5 was selected as the optimum pH value in this study since it gave highest number of theoretical plates and best sensitivity.

3.1.4. Effect of SDS concentration

SDS concentration was varied over the range of 0.05–0.15 M. In the whole mobile phases studied the separation was achieved, while a second step to be taken into consideration was to obtain a minimum analysis time. The retention times of both the drug and its degradation product decreased as the molar concentration of SDS increased. The best compromise in terms of resolution, run time, efficiency and peak symmetry was achieved upon using a mobile phase containing 0.15 M SDS.

3.1.5. Effect of type and concentration of organic modifier

To select the best organic modifier for separation of ZPC and ACP, two mobile phases containing n-propanol (10%, v/v) and pentanol (5%, v/v) were tested. The results obtained were almost the same where the drug and its degradation product were eluted at ≈4.9 and 6.4 min, respectively. n-Propanol was selected as the proper organic modifier in the present method.

Percentage of n-propanol in the mobile phase was varied over the concentration range of 8–15% (v/v). As expected, the retention of ZPC and ACP decreased as percentage of organic modifier increases. A concentration of 10% (v/v) n-propanol was chosen as the optimal concentration, where it provided a good combination of peak shape, resolution factor and analysis time.

3.1.6. Effect of column temperature

Increasing the column temperature to 45 °C resulted in split chromatographic peak of ZPC. So, the separation was carried out at room temperature.

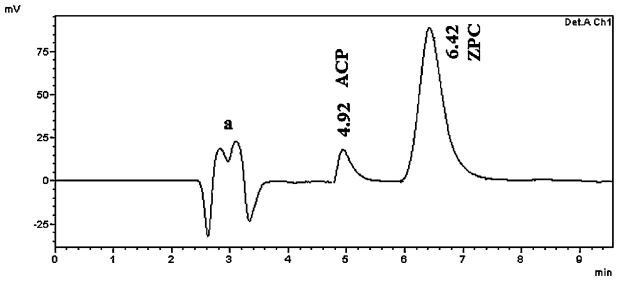

Fig. 2. Typical chromatogram showing laboratory-prepared mixture of ZPC (8 μg/mL) and ACP (2.5 ng/mL), where 'a' is the solvent front.

After theses experimental investigations, the assay was carried out using a phenyl column with mobile phase consisting of 0.15 M SDS–10% n-propanol–0.3% TEA–0.02 M H_3PO_4 at pH 3.5 and programmable fluorescence detection. Fig. 2 represents a typical chromatogram showing good resolution of ZPC and ACP (R_s = 4.19) under the optimum chromatographic conditions.

3.2. System suitability test parameters

To ascertain the reproducibility of the MLC method, system suitability tests were performed using the working standard solutions of ZPC and ACP. Resolution (R_s), theoretical plate number (N), capacity factor (k'), relative retention (α) and tailing factor (T) were measured as the criteria for system suitability testing. These results are satisfactory compared to the minimum values necessary for an acceptable method.

4. Method validation

The validity of the proposed MLC method was tested in terms of linearity, ranges, limits of detection, limits of quantification, accuracy, precision, specificity, robustness, sample solution stability and mobile phase stability.

4.1. Linearity and range

Under the above described chromatographic conditions, linear relationships were established by plotting peak area against ZPC or ACP concentrations. The concentration ranges were found to be 0.5–10.0 μg/mL for ZPC and 2.5–50.0 ng/mL for ACP. Linear

Table 2
Performance data for the proposed method for determination of ZPC in pure form.

Parameter	Results
Concentration range (μg/mL)	0.5–10.0
Limit of detection (LOD) (μg/mL)	0.16
Limit of quantification (LOQ) (μg/mL)	0.48
Correlation coefficient (r)	0.9998
Slope	2.7×10^5
Intercept	2.7×10^4
Standard deviation of the residuals ($S_{y/x}$)	2.0×10^4
Standard deviation of the intercept (S_a)	1.3×10^4
Standard deviation of the slope (S_b)	2.3×10^3
% RSD	1.56
% error (% RSD/$\sqrt{n}$)	0.63

regression analysis of the data gave the following regression equations:

$$\text{ZPC}: \quad \text{P.A.} = 2.7 \times 10^4 + 2.7 \times 10^5 C \quad (r = 0.9998)$$

$$\text{ACP}: \quad \text{P.A.} = 2.17 \times 10^4 + 3.51 \times 10^4 C \quad (r = 0.9999)$$

where P.A. is the average peak area, C is the concentration and r is the regression coefficient.

The validity of the method was proved by statistical evaluation of the regression line [25]. The fairly small values of the standard deviation of the residuals ($S_{y/x}$), of the slope (S_b) and of the intercept (S_a), and the % relative error indicate low scattering of the calibration points around the regression lines (Table 2).

4.2. Limit of quantification (LOQ) and limit of detection (LOD)

The limit of quantification (LOQ) and limit of detection (LOD) were determined according to ICH Q2 (R1) recommendations [26]. The results are summarized in Table 2.

LOQ and LOD were calculated according to the following equations [26]:

$$\text{LOQ} = \frac{10 S_a}{b}$$

$$\text{LOD} = \frac{3.3 S_a}{b}$$

where S_a is the standard deviation of the intercept of regression line and b is its slope.

Table 4
Precision data for the proposed method of determination of ZPC and ACP in pure form.

ZPC			
Conc. (μg/mL)	% found ± SD	% RSD	% error
Intra-day precision			
5.0	97.60 ± 1.52	1.56	0.90
8.0	98.71 ± 1.39	1.41	0.81
10.0	99.98 ± 1.04	1.04	0.60
Inter-day precision			
5.0	101.2 ± 1.25	1.24	0.71
8.0	101.22 ± 1.33	1.31	0.76
10.0	98.16 ± 1.43	1.45	0.84
ACP			
Conc. (ng/mL)	% found ± SD	% RSD	% error
Intra-day precision			
5.0	99.55 ± 1.47	1.48	0.85
10.0	100.71 ± 0.59	0.59	0.34
25.0	99.96 ± 0.98	0.98	0.57
Inter-day precision			
5.0	100.46 ± 0.74	0.73	0.42
10.0	100.78 ± 0.84	0.83	0.48
25.0	99.50 ± 0.92	0.92	0.53

4.3. Accuracy

The accuracy of analytical method is defined as the agreement of the results obtained by this method with the true values. To test the validity of the proposed method, it was applied to the determination of pure samples of ZPC over the range of 0.5–10.0 μg/mL. The results obtained were in good agreement with those obtained using the comparison HPLC method [3]. Using Student's t-test and the variance ratio F-test revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 3) [25].

4.4. Precision

The intra-day precision was evaluated through replicate analysis of different concentrations of the drug and degradation product in pure form within the specific working concentration ranges. Each sample was analyzed three successive times. The results are summarized in Table 4.

Table 3
Application of the proposed method to the determination of ZPC in raw material and tablets.

Matrix	Proposed method			Comparison method [3]
	Conc. taken (μg/mL)	Conc. found (μg/mL)	% found[a]	% found[a]
Raw material	0.5	0.492	98.40	101.70
	1.0	0.972	97.20	101.78
	2.0	1.983	99.15	98.95
	5.0	5.056	101.12	
	8.0	8.093	101.16	
	10.0	9.904	99.04	
$\bar{X}$ ± SD			99.35 ± 1.55	100.81 ± 1.61
t			1.32 (2.36)[b]	
F			1.08 (5.79)[b]	
Hypnor® tablets (7.5 mg ZPC/tablet)[c]	5.0	4.995	99.90	98.50
	8.0	7.990	99.88	99.01
	10.0	9.730	97.30	100.50
$\bar{X}$ ± SD			99.06 ± 1.49	99.34 ± 1.04
t			0.47 (2.78)[b]	
F			1.74 (19.0)[b]	

[a] Each result is the average of three separate determinations.
[b] Values between parenthesis are the tabulated t and F values at $P = 0.05$ [25].
[c] Nominal content of tablet = 7.43 mg/Tablet.

Similarly, the inter-day precision was evaluated through replicate analysis of the three different concentrations on three successive days. The results obtained are abridged also in Table 4.

The data presented in Table 4 indicate high precision of the developed method. Good value of the average percentage recovery and the small value of % RSD indicate the high accuracy and precision, respectively.

4.5. Selectivity

Method selectivity was evaluated by analyzing different laboratory-prepared mixtures of ZPC and ACP at concentration ratios ranging from 1:0.00025 to 1:0.001 for ZPC:ACP, respectively by using the proposed method. The average value for percent of the declared concentration of ZPC was found to be 101.22 ± 0.97. The results indicated high selectivity of the proposed method for determination of ZPC.

The proposed MLC was also found to be selective for ACP in urine samples, where, no interferences were found from the endogenous compounds of urine or from proteins.

4.6. Stability of standard solution and mobile phase

The stability of the standard solution was determined by quantification of ZPC and comparison to freshly prepared standard solution. No significant change was observed in standard solution response, relative to freshly prepared standard. When the drug was prepared (acetonitrile:water mixture, 50:50, v/v) the drug degraded very rapidly yielding ACP. So, to prevent hydrolysis, it is recommended to freshly prepare the drug in acetonitrile and keep it in the refrigerator. Similarly, the stability of the mobile phase was checked. The results obtained in both cases proved that the sample solution and mobile phase used during the assay were stable up to 7 and 2 days, respectively when kept in the refrigerator at 4 °C.

5. Applications

5.1. Application of the proposed method to quality control of ZPC in commercial tablets

The proposed MLC method was applied for the determination of ZPC in Hypnor® tablets and the results are presented in Table 3. The good percentage recoveries with small SD value confirm the suitability of the proposed method for the routine determination of this compound in commercial tablets. As can be seen, the values found agreed well with those declared by the manufacturer. Statistical analysis of the results obtained by the proposed method and those given by the comparison method was performed using Student's *t*-test and the variance ratio *F*-test. As illustrated in Table 3, the calculated values did not exceed the theoretical ones, indicating no significant difference in the performance of the compared methods regarding accuracy and precision. Fig. 3 shows the chromatogram obtained for Hypnor® tablets analyzed under the conditions selected previously.

The developed method was found to be able to detect and quantify ACP in ZPC tablets over the range of 0.025–10%, so it can be applied for purity testing of ZPC tablets within the BP pharmacopeial limit (0.5%) [2].

5.2. Application of the proposed method to the determination of ACP in human urine as a marker for ZPC intake

A practical and important consequence of ZPC instability is its degradation in urine samples when they are not properly conserved [17]. This instability of ZPC can be used as a mean for the detection of its intake through the determination of its degradation product in urine. The applicability of the developed MLC method for the detection of ACP in urine samples was examined. MLC allows biological samples to be analyzed without prior pretreatment steps for elimination of interfering substances. Urine samples were injected directly into the chromatographic system after 1:10 dilution with the selected mobile phase at pH 3.5. Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs. Proteins are solubilized and washed harmlessly away, eluting with the solvent front rather than precipitating into the column [27]. The background signal of urine samples, due to the proteins and several endogenous compounds can seriously affect the detection of the studied drug. Moreover, the injection of a large number of urine samples can produce damage to the packing material, thus shortening the life of the column, or can necessitate frequent regeneration of the stationary phase. However, dilution of the urine sample before its injection reduced the width of the protein band and also benefiting the column by increasing its useful life. Other advantages are that the micellar mobile phases are nontoxic, non-flammable, biodegradable and relatively inexpensive, in comparison to aqueous–organic solvents [27].

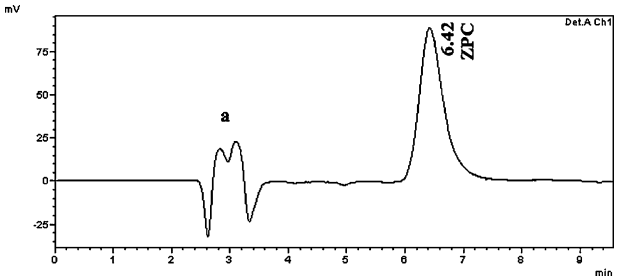

Fig. 3. Typical chromatogram showing ZPC (8.0 μg/mL) in Hypnor® tablets, where 'a' is the solvent front.

Fig. 4 represents typical chromatograms for blank and spiked urine samples. No interference is observed and the ACP peak is well separated from the protein band. Table 5 represents the results of determination of ACP in spiked human urine. The obtained results are promising that it can be applied for the determination of ACP in real human urine sample following ingestion of ZPC after a

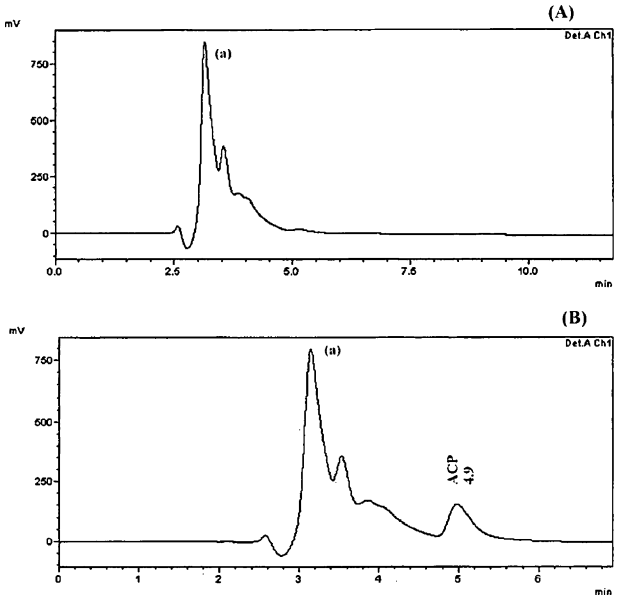

Fig. 4. Typical chromatograms showing: (A) urine blank and (B) urine sample spiked with ACP (0.05 μg/mL), where 'a' is the urine peak.

Table 5
Application of the proposed method to the determination of ACP in spiked human urine.

Parameter	Amount added (ng/mL)	Amount found (μg/mL)	% found[a]
Spiked urine	5.0	4.70	94.0
	10.0	10.40	104.0
	50.0	50.00	100.0
Mean found (x)			99.33
±SD			5.03
% RSD			5.07
% error			2.91

[a] Each result is the average of three separate determinations.

short alkaline hydrolysis treatment to convert any residual cyclopyrrolone moiety to the decomposition product (ACP) [17].

6. Conclusion

The proposed MLC method represents an accurate, precise and reliable stability-indicating assay method for ZPC. The developed method was able to discriminate between ZPC and its degradation product, ACP. The method is highly sensitive allowing the determination of ACP in ZPC even in very low concentration down to 2.5 ng/mL. The developed method was applicable for assay and purity testing of ZPC in bulk and pharmaceutical formulations. The proposed MLC was used to detect ACP in spiked human urine as a parameter of ZPC intake with no need for extraction or pretreatment steps.

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