Voltammetric Behavior of Zaleplon and Its Differential Pulse Polarographic Determination in Capsules

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In this work both the electrochemical behavior and the analysis of the hypnotic pyrazolopyrimidine derivative zaleplon were studied. Zaleplon in ethanol–0.1M Britton Robinson buffer solution (30–70) showed 2 irreversible, well-defined cathodic responses in the pH range of 2–12 using differential pulse polarography (DPP), tast polarography, and cyclic voltammetry. From chronocoulometric studies, it was possible to conclude that one electron was transferred in each reduction peak or wave. For analytical purposes, the DPP technique working at pH 4.5 for peak I was selected, which exhibited adequate repeatability, reproducibility, and selectivity. The recovery was 99.97 ± 1.52%, and the detection and quantitation limits were 5.13×10^{-7}M and 1.11×10^{-6}M, respectively. The DPP method was applied successfully to the individual assay of capsules in order to verify the content uniformity of zaleplon. Treatment of the sample is not required because the excipients do not interfere, the method is not time consuming, and it is less expensive than column liquid chromatography.

Zaleplon, *N*-[3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl)phenyl]-*N*-ethylacetamide (Figure 1), is a pyrazolopyrimidine derivative hypnotic with a chemical structure that is unrelated to benzodiazepines or other known hypnotics. It binds selectively to the benzodiazepine type-1 site on the γ-aminobutyric acid subtype-A($GABA_A$)/chloride ion channel complex (1–3).

Zaleplon is lipophilic with an approximate 1.4 L/kg volume of distribution after intravenous administration. It is approximately 60% plasma protein-bound independent of its concentration over the range of 10 to 1000 ng/mL (4, 5) After oral administration, zaleplon is extensively metabolized, with less than 1% of the dose excreted unchanged in urine. All metabolites of the zaleplon are inactive. Zaleplon is primarily metabolized by aldehyde oxidase to form 5-oxozaleplon. To a lesser extent, it is metabolized by CYP3A4 to form desethylzaleplon, which is converted, reportedly by aldehyde oxidase, to 5-oxo-desethylzaleplon. Then, these oxidative metabolites are converted to glucuronides and eliminated in urine (6).

Considering that zaleplon is a novel drug, few analytical methods for its determination have been described. For the simultaneous determination of zaleplon and its metabolites, different liquid chromatographic (LC) methods with both ultraviolet (UV) and fluorescence detection have been described (7–9). LC with fluorescence detection has been also applied in preclinical studies (7) and for the determination of zaleplon in breast milk after oral administration to lactating women (10). Also, the development and validation of an LC/electrospray ionization-mass spectrometry (MS) assay for the determination of zaleplon in human plasma (11) and the determination of zaleplon and zolpidem by LC/turbo-ionspray-MS applied to forensic cases (12) have been described. Recently, the separation and identification of zaleplon metabolites in human urine using capillary electrophoresis with laser-induced fluorescence detection and LC/MS have been published (13).

In spite of the fact that the redox behavior of zaleplon can be key knowledge in order to follow its metabolic or degradative mechanism, at the current time there are no reported studies of the electrochemistry of zaleplon. Furthermore, an official method for the determination of this drug in pharmaceutical forms has not yet been described in any pharmacopoeia. Considering this lack of knowledge, we were interested in investigating the electrochemistry of zaleplon in order to develop a differential pulse polarography (DPP) method that can determine this drug in pharmaceuticals.

Experimental

Reagents and Drugs

(**a**) *Zaleplon*.—100% chromatographically pure, was supplied by Prater Laboratories (Santiago, Chile).

(**b**) *Commercial capsules of Plenidon*®.—Declared amount/capsule of 10 mg zaleplon, obtained commercially from Drugtech Laboratories (Santiago, Chile).

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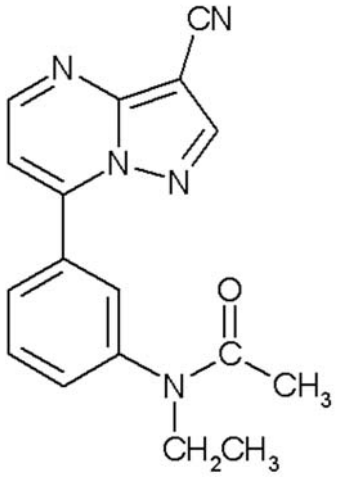

Figure 1. Chemical structure of zaleplon.

(**c**) *Reagents*.—Analytical grade unless indicated otherwise: sodium hydrogen phosphate, phosphoric acid, and acetonitrile LC grade (Mallinckrodt, Phillipsburg, NJ) were used.

(**d**) *Water*.—Deionized, prepared in the laboratory using ion exchange columns; Milli-Q system (Milford, MA).

Preparation of Solutions

(**a**) *Buffer solutions*.—0.1M Britton-Robinson buffer (acetic acid–boric acid–phosphoric acid) was used for polarographic experiments; the desired pH was adjusted with concentrated solutions of NaOH or HCl. For LC, a 0.01M phosphate buffer solution (disodium hydrogen phosphate anhydrous salt adjusted to pH 3.0 with phosphoric acid) was used.

(**b**) *Stock drug solution*.—Ca 6.2 mg zaleplon was dissolved and diluted to 10 mL with ethanol to obtain a final concentration of ca 2×10^{-3}M zaleplon. The solution was protected from light by using amber glass materials.

(**c**) *Working solution*.—An aliquot of the stock solution was diluted to 10 mL with acetonitrile–phosphate buffer solution (pH 3.0, 0.01M; 35 + 65, v/v) for LC, or with ethanol–Britton-Robinson buffer solution (0.1M; 30 + 70, v/v) for both UV-Visible (Vis) spectroscopy and DPP.

Apparatus

(**a**) *Voltammetric analyzer*.—DPP, tast polarography (TP), differential pulse voltammetry (DPV), and cyclic voltammetry (CV) experiments were performed with a Metrohm Model 693 VA-processor (Herisau, Switzerland), equipped with a Model 694 VA-stand, 25 mL thermostated measuring cell with a dropping mercury electrode (DME), hanging mercury drop electrode (HMDE), or glassy carbon rotating electrode (GCE; slashed circle = 2 mm) as the working electrode; platinum wire counter electrode; and calomel reference electrode. The operating conditions were sensitivity, 2.5–10 μA; drop time, 1 s; potential range, 0–1800 mV; Δ Ep, –4 mV; pulse retard, 40 ms; and pulse height, –50 mV. When a glassy carbon was used as working electrode, the surface was polished with alumina (14).

(**b**) *Chronocoulometric studies*.—The assays were performed using a totally automated Bioanalytical System CV-50W, composed of a 10 mL electrolysis cell; a mercury pool as the working electrode; and Ag/AgCl 3M KCl and Pt reference and auxiliary electrodes, respectively. Zaleplon (2×10^{-5} mole) was dissolved in 10 mL ethanol–Britton-Robinson buffer (0.1M; 30 + 70, v/v). The electrolysis potentials were –850 and –1400 mV for pH 3 and 7, respectively. Experiments at each pH were performed in triplicate.

(**c**) *LC*.—Measurements were performed by using a Waters (Milford, MA) assembly equipped with a Model 600 controller pump and a Model 996 photodiode array detector. The acquisition and treatment of data were made with the Millenium Version 2.1 software. The chromatographic column was Bondapak/Porasil C18, 3.9 × 150 mm. A C18 Bondapak guard column (30 × 4.6 mm) was used. The injector was a 20 μL Rheodyne valve. UV detection at 356 nm was used, and the column was kept at constant temperature using a Waters column heater cartridge Model 600.

Isocratic elution was performed with a mobile phase consisting of acetonitrile–phosphate buffer (pH 3.0; 0.01M; 35 + 65, v/v). The flow rate was 1.0 mL/min, and the working temperature was kept constant at 30° ± 1°C. With these conditions, zaleplon exhibited a retention time of 4.74 ± 0.08 min.

(**d**) *Spectrophotometer*.—Spectrophotometric measurements were performed with an ATI Unicam model UV3

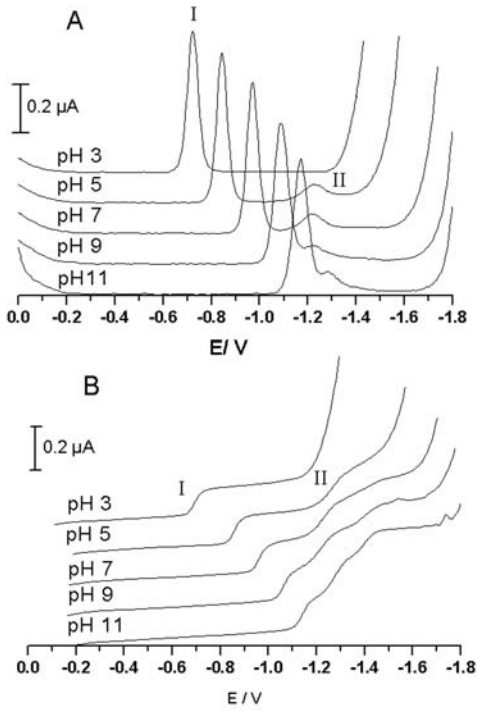

Figure 2. (A) Differential pulse and (B) tast polarograms of 0.1mM zaleplon in ethanol–Britton-Robinson buffer (0.1M; 30 + 70, v/v) at different pH values.

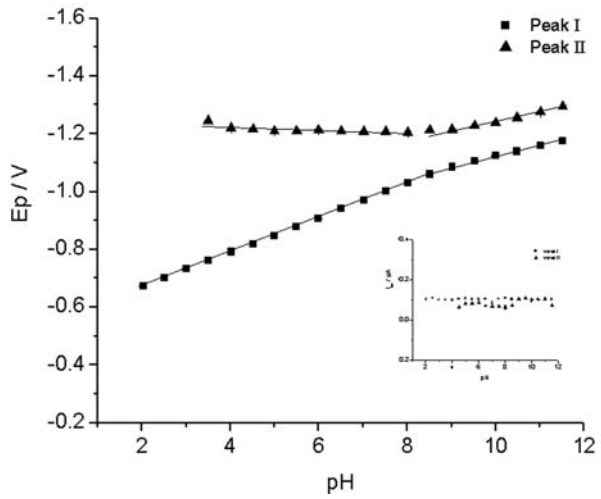

Figure 3. Peak potential and limiting current (insert) behavior at different pH values of 0.1mM zaleplon in ethanol–Britton-Robinson buffer (0.1M; 30 + 70, v/v).

spectrophotometer (Cambridge, UK), using a 1 cm quartz cell and equipped with an Intel 486 computer with Vision acquisition and treatment software.

Analytical Procedures

(**a**) *Calibration curve preparation*.—For polarography and UV spectroscopy, the zaleplon stock solution was diluted with ethanol–Britton-Robinson buffer (pH 4.5, 0.1M; 30 + 70, v/v) to prepare working solutions ranging between 3×10^{-6} and 3×10^{-5}M.

For LC the zaleplon stock solution was diluted with mobile phase, and working solutions ranging between 3×10^{-6} and 3×10^{-5}M were prepared. The solutions were injected and chromatographed according to the working conditions previously given. The UV detector was set at 232 nm.

Excipients (cornstarch, magnesium stearate, lactose, sodium lauryl sulfate, and microcrystalline cellulose) were added to the drug to prepare synthetic samples for recovery studies, according to manufacturer's batch formulas for 10.0 mg zaleplon capsules.

(**b**) *Individual capsules assay procedures*.—For the polarography study, no fewer than 10 commercial capsules of zaleplon (Plenidon amount declared 10 mg zaleplon/capsule) were used. The content of each capsule was independently suspended in 10 mL ethanol with sonication to ensure the complete dissolution of the drug and diluted to a final volume of 100 mL with the same solvent. A 1 mL aliquot of each solution was taken and diluted to 25 mL with ethanol–Britton-Robinson buffer solution (pH 4.5, 0.1M; 30 + 70, v/v) to obtain a zaleplon concentration of 6.2×10^{-5}M. Each sample solution was transferred to a polarographic cell, bubbled with nitrogen for 5 min, and measured at least twice from –700 to –1000 mV. The mg amount of zaleplon in the sample solution was calculated from the prepared standard calibration graph.

For the UV spectroscopy and LC studies, no fewer than 10 commercial capsules of zaleplon (Plenidon, amount declared 10 mg zaleplon/capsule) were used. The content of each capsule was independently suspended in 10 mL ethanol with sonication to ensure the complete dissolution of the drug and diluted to a final volume of 100 mL with ethanol–Britton-Robinson buffer solution (pH 4.5, 0.1M; 30 + 70, v/v) for UV spectroscopy or with the mobile phase for LC. Each of the above solutions was centrifuged for 10 min at 4000 rpm, and then a 0.5 mL aliquot of the supernatant was taken and diluted to 10 mL with the appropriate solvent to obtain solutions of ca 7×10^{-5}M zaleplon. The mg amount of zaleplon in the sample solution was calculated from the corresponding prepared standard calibration curve.

(**c**) *Selectivity studies (15).—(1) Hydrolysis*.—Ca 4 mg zaleplon was dissolved in 5 mL ethanol in 3 separate 10 mL distillation flasks; 5 mL water was added to the first for neutral hydrolysis, 5 mL 0.1M HCl to the second for acid hydrolysis, and 5 mL 0.1M NaOH to the third for basic hydrolysis. Subsequently, each solution was boiled for 1 h at reflux. *(2) Photolysis*.—*(a) Zaleplon raw material*.—Ca 4 mg was put in a black box and irradiated with a UV light (UV Black-Ray; San Jose, CA) longwave ultraviolet lamp, UVP model B 100 AP, 50 Hz, 2.0 A with a 100 W Par 38 mercury lamp equipped with a 366 nm filter at a distance of 15 cm for 8 h (1.2×10^{19} quanta/s, determined by using a potassium ferrioxalate chemical actinometer (16). *(b) Zaleplon ethanol solution*.—10 mL 1×10^{-3}M zaleplon ethanol solution was bubbled for 2 min with nitrogen, transferred to a black box, and irradiated with UV light as the raw material. *(3) Thermolysis*.—Ca 4 mg zaleplon raw material was heated at 105°C for 5 h.

An appropriate volume of each solution obtained from the degradation trials (solids were dissolved in 10 mL ethanol) was taken and divided to a final volume with

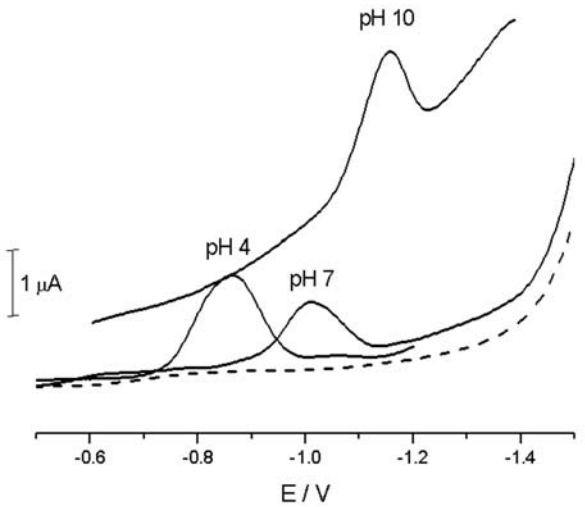

Figure 4. Differential pulse voltammograms with a glassy carbon electrode of 0.1mM zaleplon in ethanol–Britton-Robinson buffer (0.1M; 30 + 70, v/v) at 3 different pH values.

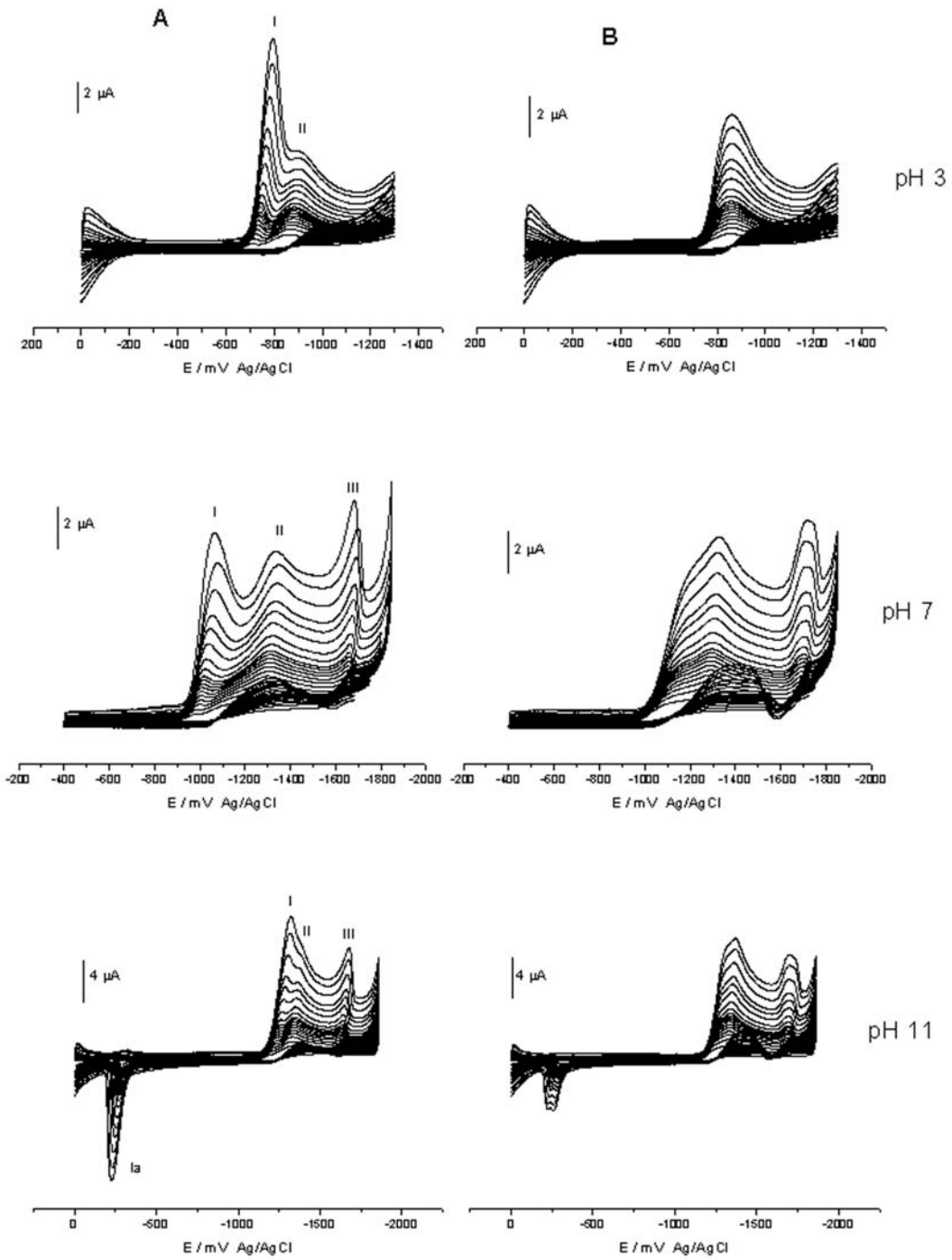

Figure 5. Cyclic voltammograms of (A) 1mM zaleplon in ethanol–Britton-Robinson buffer (0.1M; 30 + 70, v/v) and (B) plus 0.01% Triton at pH 3, 7, and 11 at different sweep rates from 0.1 to 6 V/s.

Britton-Robinson buffer solution (pH 4.5, 0.1M) to obtain a theoretical concentration of 6×10^{-5}M zaleplon. Samples from these studies were stored at –20°C and protected from light prior to polarography and LC analysis. Each sample was analyzed in duplicate.

(**d**) *Statistic analysis*.—Comparison between the different techniques, as well as the comparison with standard deviations, was performed by means of Student's *t*-test using a significance limit of 95% confidence (17, 18).

Results and Discussion

Cathodic Behavior

Zaleplon in ethanol–Britton Robinson buffer solution (0.1M; 30 + 70, v/v) gave a well-defined cathodic response in the pH range of 2–12 when studied by both DPP and TP (Figure 2). In Figure 2A, typical differential pulse polarograms of zaleplon at different pH values are shown. As can be seen, at acidic pH (<3.5) a well-resolved peak appeared that was shifted towards more negative potentials as the pH

Table 1. Analytical parameters for the developed methods

Parameter	DPP	UV (232 nm)	LC-UV (232 nm)
Repeatability, CV (%)[a]	1.97	1.30	1.65
Reproducibility, CV (%)[a]	1.99	1.35	1.67
Recovery (%) ± SD[b]	99.97 ± 1.52	97.07 ± 1.78	94.33 ± 1.16
Concentration range, M	1×10^{-6}–3×10^{-5}	1×10^{-6}–3×10^{-5}	1×10^{-6}–3×10^{-5}
Calibration curve	Ip(μA) = –0.0035 – 7842.2243 × C(M) (n = 10, r = 0.9996)	AU = –0.0351 + 39656.9 × C(M) (n = 10, r = 0.9992)	AUC = 8877.6504 + 4.7957 × 10^{10} × C(M) (n = 10, r = 0.9996)
Detection limit, M	5.13×10^{-7}	2.52×10^{-7}	1.05×10^{-7}
Quantitation limit, M	1.11×10^{-6}	8.4×10^{-6}	3.49×10^{-7}

[a] Concentration level of 5×10^{-5}M; CV = coefficient of variation.

[b] Average on a concentration level of 5×10^{-5}M; SD = standard deviation.

increased (Peak I). At pH higher than 3.5 a second peak was observed at more negative potentials (Peak II). Finally, at basic pH an overlapping of both peaks was observed. By using TP (Figure 2B), behavior similar to DPP was observed.

In Figure 3, a plot of the peak potential vs pH is shown. The peak potential of Peak I is seen to be linear with pH, and 2 zones can be observed with breaks at pH 9 (slopes of 59.7 mV/pH, pH 2–9, and 36.2 mV/pH, pH 9–11.5). This break presumably is due to a change in the protonation-deprotonation process of the electroactive molecule. For Peak II, Ep remains pH independent between pH 2–9, and above pH 9 it becomes pH dependent with a slope of 31.7 mV/pH (pH 9–11.5). The peak currents of both peaks remain independent of pH in the whole range studied, and the peak current of Peak I corresponds to approximately 6 times the peak current of Peak II (data not shown).

In the tast mode, similar behavior in terms of Ep vs pH was observed for half wave potentials. The limiting current of both Waves I and II exhibited an independent pH behavior as in the DPP mode (Figure 3), but in this case the limiting current of Wave I has the same intensity as Wave II, indicating that the same number of electrons are transferred in each case.

By DPV on a glassy carbon electrode, zaleplon generates only one reduction peak in the pH range studied. This peak is pH dependent and shifted towards more negative potential as the pH increases, with a slope of 45.2 mV/pH. The DPV signals are wider than those observed in the DPP mode, and at alkaline pH values the signal is overlapped with the solvent discharge (Figure 4).

By linear CV at pH 3, 7, and 11, irreversible waves at all sweep rates studied were observed (Figure 5A), and larger signals than those observed by pulse techniques were also present. Analyzing the relationship between peak current and sweep rate (log Ip vs log υ graph), a strong adsorption phenomenon was identified because slopes of 0.887, 0.862, and 0.875 were determined for signal I at pH 3, 7, and 11, respectively. On the other hand, for signal II the slopes were 0.468, 0.504, and 0.532 for pH 3, 7, and 11, respectively; a slope value near 0.5 indicates that the electrode process is diffusion-controlled; therefore, signal II is not affected by the

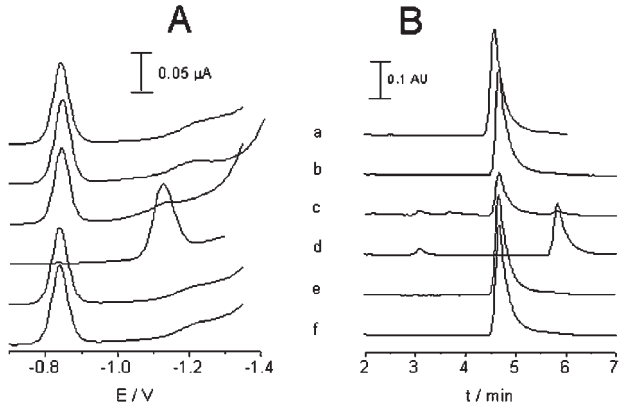

Figure 6. DPP (A) and LC (B) results for zaleplon degradation trials: (a) standard, (b) neutral hydrolysis, (c) acidic hydrolysis, (d) basic hydrolysis, (e) photolysis, and (f) thermolysis.

Table 2. Individual capsule assay of zaleplon[a]

	Method		
Parameter	DPP	UV	LC-UV (356 nm)
Average (n = 10)	9.93	9.58	9.38
SD	1.82	1.79	1.68
CV, %	1.83	1.86	1.79
t_{exp}[b]	—	0.433	0.878
F_{exp}[c]	—	1.034	1.174

[a] Declared amount/capsule = 10.0 mg zaleplon.

[b] t_{table} (P = 0.05) = 2.1.

[c] F_{table} (P = 0.05) = 4.026.

adsorption. For peak III, slopes of 0.574 and 0.641 for pH 7 and 11, respectively, were determined, indicating diffusion and adsorptive control. Finally, only one anodic peak was detected, at pH 11 (peak Ia), which indicates strong adsorption control based on a slope of 0.783. All the adsorptive peaks can be suppressed by adding the surfactant Triton to the solution (Figure 5B).

In order to determine the number of electrons transferred in the electroreduction of zaleplon, the relationships between charge and electrolysis time at pH 3 and 7 were evaluated. The number of electrons involved in the electrodic process was 1.13 ± 0.02 for E = –850 mV and 2.03 ± 0.02 for E = –1400 mV. As the second electrolysis (E = –1400 mV) involves both electrode processes, it is possible to conclude that 1 electron is transferred in each reduction peak.

According to the above results and the information for related molecules described in the literature (19, 20) we can conclude that the electro-reduction might involve 2 successive 1-electron transfer steps to the pyrimidine moiety, probably generating unstable intermediates that might undergo a rapid dimerization reaction.

The difference with the literature is that when a pyrimidine was studied, two 1-electron steps only at pH < 5 were found (19), but in our work the same behavior at all pH values studied (2–12) was observed. This difference is probably because zaleplon is a cianopyrazolopyrimidine derivative.

In other work in which the electrochemical reduction of various pyrazolo[1,5-α]pyrimidines was studied between pH 0–7, the polarographic behavior in hydroorganic medium was different according to the nature and the location of the substituents on the pyrimidine ring, which is solely affected by the reduction. For example, with the presence of a phenyl substituent on the pyrimidine ring, like zaleplon, two 1-electron cathodic waves were observed, whereas with 5,7-dimethyl derivatives, a single 1-electron cathodic wave was observed (20).

Analytical Applications

For analytical purposes we selected the DPP technique because the mercury electrode exhibited better reproducibility than the glassy carbon electrode; the selected signal was peak I at pH 4.5. The Ip for peak I varied linearly with zaleplon concentration between 3×10^{-6} and 3×10^{-5}M. Calibration curves were made for both peaks, but when peak II was used low sensitivity and sensibility were obtained (slopes of 7842 μA/M vs 587 μA/M for peaks I and II, respectively). Within-day and inter-day reproducibility were adequate, with relative standard deviation (RSD) values lower than 2%. In Table 1, the analytical parameters are summarized.

In order to check our proposed method for selectivity, we tried different degradation pathways for zaleplon. Selectivity is a parameter that describes the capacity of the method to produce a signal due only to the presence of the analyte (zaleplon) and, consequently, free of other interferences, such as degradation products, metabolites, or pollutants. In order to prove the selectivity, we have conducted the following trials: hydrolysis (acidic, basic, and neutral); photolysis, and thermolysis (15).

When a zaleplon solution was exposed to either basic or acidic hydrolysis, the polarographic peak disappeared or diminished, respectively, and a new peak appeared near to –1150 mV (Figure 6A). This behavior was also found by LC (Figure 6B), with new chromatographic peaks appearing and the UV spectra of the new signals not corresponding with the UV spectrum of zaleplon.

In order to check the selectivity of possible photo-decomposition products, zaleplon was exposed to 366 nm UV light for 8 h. In this experiment, zaleplon's peak remained unaltered. Similar behavior was obtained when zaleplon was submitted to the thermolysis test.

Based on the results obtained in the selectivity trials, it can be concluded that the proposed DPP method is sufficiently selective to be applied to zaleplon quantitation.

In order to assess the precision and accuracy of the developed method, a recovery study was performed. These results (Table 1) reveal that the method has adequate precision and accuracy and, consequently, can be applied to the determination of zaleplon in a commercial dosage form (capsules). Also, from these experiments we can conclude that the typical excipients included in the drug formulation (cornstarch, magnesium stearate, lactose, sodium lauryl sulfate, and microcrystalline cellulose) do not interfere with the selectivity of the method, and previous separation or extractions are not necessary.

Finally, the proposed DPP method was applied successfully to the assay of individual capsules in order to verify the content uniformity of zaleplon; for comparative purposes, both UV and LC analyses were also performed (Table 2). As can be seen, the content for all assayed capsules is within ± 6.5% of the claimed amount, fulfilling the Pharmacopoeia requirement for uniformity content of capsules that permits a ± 15% tolerance in this kind of dosage form (21).

Comparing the results obtained in the uniformity content test by applying the *F* test (variance proportion) and then the Student's *t*-test ($P = 0.05$, $n = 10$) between DPP and the comparative methods, it is possible to conclude that no significant differences exist between them and that they are statistically equivalent (Table 2).

Finally, we can conclude that the developed DPP method is an adequate tool for zaleplon determination in pharmaceutical dosage forms because it exhibits an adequate accuracy, reproducibility, and selectivity. Furthermore, pre-treatment of the sample is not required, the method is not time consuming, and it is less expensive than LC.

Acknowledgments

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