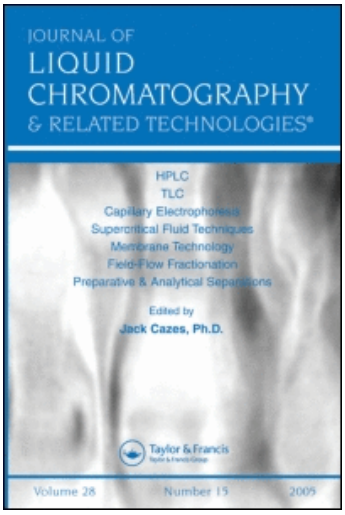

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Optimization by Factorial Design of a Capillary Electrophoresis Method for the Chiral Resolution and Determination of Zopiclone and Its Synthesis Precursor

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Abstract: A rapid, simple and accurate capillary electrophoretic method has been developed for the determination of zopiclone and its synthesis precursor, zopiclone II, by a factorial design. The compounds are separated in a fused silica capillary (48.5 cm length, 40 cm effective length, 50 μm inner diameter) with UV detection at 215 nm. Separation was achieved with a 60.2 mM phosphate buffer containing 20 mM β-cyclodextrin and 1 M of urea, adjusting the pH to 2.0 and using diazepam as an internal standard. The method was validated in accordance with the International Conference on Harmonization guidelines, appropriate linearity and precision being observed for all compounds. The recoveries obtained ranged between 97.6% and 100.7%. This method is sensitive (detection limits ranged between 2.1 and 7.2 mg L^{-1}) and selective to quantify the enantiomers of zopiclone and its synthesis precursor and could be used to evaluate the chiral intermediates and the enantiomeric purity of the final product.

Keywords: Capillary electrophoresis, Chiral, Cyclodextrin, Experimental design, Zopiclone

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INTRODUCTION

Sleep disorders can be classified into roughly 90 different types on the basis of features of the symptoms, cause of the disease, etc.[1] Most of the medicines for treating these disorders nowadays are benzodiazepines and their derivatives. Non-benzodiazepines, such as zolpidem, zopiclone (ZPN), etc., which are comparatively new hypnotics, are structurally different from benzodiazepines, but exhibit the same activities as benzodiazepines through benzodiazepine receptors.[2]

ZPN (4-methyl-1-piperazinecarboxylic acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]-pyrazin-5-yl ester) (Figure 1a) and its optically pure enantiomers are effective hypnotics that have been reported to be useful in the treatment of sleep disorders of various aetiologies and other types of diseases (epilepsy, anxiety, aggressive behaviour, muscle tension, behavioural disorders, depression, schizophrenia, and endocrine disorders).[3,4] Some studies suggest that zopiclone undergoes stereo conversion and that it is stereo-specifically distributed to the brain.[5] The recommended dose in patient could thus be reduced to decrease the adverse effects of the enantiomeric form without therapeutic activity, while maintaining the beneficial effect of the drug. The (*S*)-enantiomer of the hypnotic drug ZPN is currently being reviewed by the FDA as a potential new treatment for insomnia.[6] Results of recent, well-designed nightly clinical trial shows that this optically pure enantiomer produces significant improvements in sleep latency, sleep duration, and sleep quality in patients with chronic insomnia without the risk of next-day impairment, tolerance, or rebound effects. (*S*)-ZPN showed sustained efficacy and good tolerability. In this context, analytical methods to determine the enantiomeric purity of the final product need to be developed.

Some traditional techniques for the determination of ZPN in tablets include polarographic methods.[7,8] The literature reports rapid, simple,

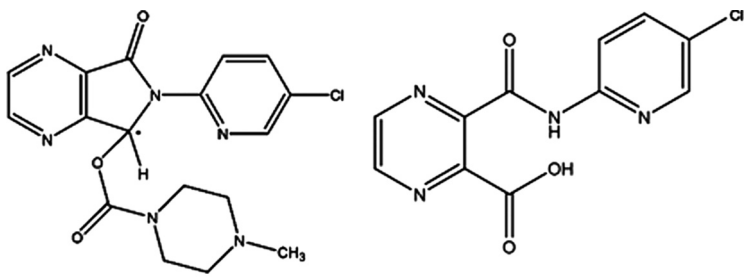


Figure 1. Structures of (a) zopiclone (ZPN) and (b) intermediate (ZPN II).

and accurate chromatography (HPLC) methods, such as the method described by Mannaert et al.[9] using a Chiralpak AS column for the separation and purification of ZPN enantiomers, or the method described by Bounine et al.[10] which enables the determination of ZPN in tablets using a LiChrospher-60 RP Select B column and UV detection. In other studies, some research was done with CE for separation of enantiomeric pairs of market drugs upon addition of β-cyclodextrin (β-CD) or hydroxypropyl-β-CD or their mixture to the run buffer.[11] The action around enantiomers entrapped in the matrix and the forming action of the inclusion-complexation between enantiomers and cyclodextrin (CD) were discussed. Chiral CE has been increasingly used in enantiomeric resolution because of its simplicity and rapidity. Furthermore, this technique is highly versatile with a minimal use of expensive chiral reagents.[12]

Several methods for the analysis of the enantiomers using CE with CD as the chiral selector have been reviewed by Bernhard Koppenhoefer et al.[13] including some references of ZPN. However, to our knowledge, the analysis of the enantiomers of ZPN and his precursor during their synthesis has not been studied.

In order to avoid the time consuming univariate procedures and to study, simultaneously, the effect of individual variables and their combined effects, various chemometric experimental designs have been employed for the multivariate optimization of CE methods.[14] Thus, fractional factorial designs,[15,16] factorial design,[17–19] central composite design,[20,21] Plackett-Burman design,[22,23] overlapping resolution mapping,[24–27] and a weighted variable-size simplex algorithm[28] were used.

In the present study, a simple and selective CE assay for the analysis of the enantiomers of ZPN and its synthesis precursor zopiclone II (ZPN II) (Figure 1b) was developed using a factorial design. The validation parameters stated by the International Conference on Harmonization guidelines were followed. The results were compared with those obtained by the well established HPLC method.[9]

EXPERIMENTAL

Reagents and Standards

4-methyl-1-piperazinecarboxylic acid 6-(5-chloro-2-pyridinyl)-6, 7-dihydro-7-oxo-5H-pyrrolo[3,4-b]-pyrazin-5-yl ester (ZPN), intermediate ZPN II, and diazepam were kindly supplied by Asturpharma (Silvota-Llanera, Spain). β-cyclodextrin, heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin were purchased from Sigma (St. Louis, MO, USA), phosphoric acid, potassium phosphate and urea

were obtained from Merck (Darmstadt, Germany), sodium hydroxide was supplied by Probus (Badalona, Spain) and acetonitrile was purchased from Romil (Loughborough, UK). Milli-Q water (Millipore, Milford, MA, USA) was used throughout.

Standard Solutions

Stock solutions of ZPN, ZPN II and diazepam (I.S.) were prepared at a concentration of 1.0 mg mL^{-1} by dissolving the appropriate amounts of the substances in acetonitrile. The solutions were stored at 4°C and protected from light. All solutions proved to be stable for more than 1 month under these conditions. The working solutions were obtained by adequate dilution of the diazepam, ZPN and ZPN II standard solutions in acetonitrile. All the solutions were filtered through 0.22 μm syringe filters prior to use.

Samples

Different samples of ZPN and ZPN II were synthesized and supplied by Asturpharma (Silvota-Llanera, Spain). The commercial sample was obtained from a local chemist.

Apparatus and Conditions for the CE

A Hewlett-Packard 3D CE system (Waldbronn, Germany), equipped with a UV-Vis photodiode array detector and HP ChemStation Software, was used. Determination of the compounds was carried out at 215 nm and identification was performed using their migration time and UV spectra.

Fused silica capillary tubes (48.5 cm length, 40 cm effective length, 50 μm inner diameter) were supplied by Merck (Darmstadt, Germany). The capillary was conditioned prior to its first use by flushing with 1 M NaOH for 20 min and then with water for 10 min. In the optimised method, the capillary was washed with NaOH 0.1 M for 2 min and then filled with the separation buffer for 3 min. The pH of the running buffer (phosphate 60.2 mM with 20 mM β-CD and 1 M urea) was adjusted to 2.0 with phosphoric acid. The final solution was filtered through a 0.45 μm membrane filter and degassed in an ultrasonic bath prior to use. The number of separations that can be made with the same set of separation vials was estimated to be 10.

The sample was introduced from the anodic end of the fused-silica capillary by a 3.5 s hydrodynamic injection with a pressure of 50 mbar.

The separations were performed using "normal" polarity (the cathode was located on the detector side). The capillary was thermostated at 25°C and constant voltage of 30 kV was applied.

Quantification was carried out by the internal standard method. Different volumes, ranging between 1.0 and 6.0 mL, of the stock solution of ZPN and 2.0 mL of the stock solution of diazepam (I.S.) were transferred into a 10.0 mL volumetric flask. This was then raised up to volume with acetonitrile. Other solutions were prepared by appropriate dilution of this solution. A volume of 0.5 mL of each sample was transferred to the vial and was then injected into the CE system. Each measurement was repeated three times for each concentration.

Apparatus and Conditions for the HPLC

HPLC analyses were performed with a Shimadzu HPLC system (Duisburg, Germany) equipped with two LC-10AD pumps, a UV-Vis SPD-M10AD photodiode array detector. The column employed was a Chiralpak AS column (250×4.6 mm, 10 μm) from Daicel Chemical Industries. The HPLC analysis was conducted using the method described by Mannaert et al.[9] The column temperature was set at 30°C and UV detection at 300 nm. The mobile phase consisted of a mixture of hexane: ethanol:diethylamine (60/40/0.1). The flow rate was set at 0.7 mL min^{-1}.

Experimental Design

Factorial design[18] is an experimental design involving simultaneous alteration of all parameters according to a predefined matrix of experiments. They are well adapted to the determination of the relative importance of each variable in comparison to the estimated responses.

Capillary (length and inner diameter), injection, temperature, running buffer (type and pH), chiral selector (type and concentration), and applied voltage are factors to take into account in the chiral CE method optimization because every one of them influences the separation. Moreover, resolution and migration times are response variables to evaluate electrophoretic performance.

Based on initial experiments, capillary, injection, temperature, running buffer type, and chiral selector type were selected. Next, four quantitative factors (β-CD concentration, background buffer pH and concentration, and voltage) that are considered to have most influence on the separation were evaluated using an experimental design supported by the Statgraphics statistical package. Levels of each factor for finding out the optimum values and responses were selected, taking into account

the chemical and physical properties of the substances and our previous experiences. The selected response variables were the resolution between ZPN enantiomers, the migration time of the *S*-ZPN, and the resolution between *S*-ZPN and diazepam. Preliminary studies suggest that ZPN II was well separated in any case.

RESULTS AND DISCUSSION

Firstly, the capillary dimensions, the running buffer, the type of CD, and temperature were selected. A 50 μm × 48.5 cm fused-silica capillary (40 cm to the detection window) and a phosphate buffer were chosen. Among the different neutral CDs tested as chiral selectors, β-CD being selected based on their biggest capacity to separate the enantiomers of zopiclone. The increase in the β-CD concentration is limited by the solubility of this additive in aqueous buffers (approximately 16 mM, at room temperature and neutral pH). Urea is an excellent additive for increasing the solubility;[11] thus, we added it to the buffer to a final concentration of 1 M. Using this additive, the total β-CD was dissolved, even when a 20 mM concentration was used. Temperature was not included in the optimization process because, in previous assays, it was found that small variations around room temperature have a little effect on resolution; as a consequence it was fixed at 25°C.

The Factorial Design Method

Once the running buffer, the capillary dimensions, the type of CD, and the temperature were selected and, to evaluate the influence on the separation of the factors (pH and concentration of running buffer, voltage and the β-cyclodextrin concentration), their intervals and the response variables (resolution and migration time) a two-level full factorial design (2^4) was used (see Table 1). The low and high values were obtained from the preliminary investigations. As the result of the adopted factorial design, a total of 17 experiments, shown in Table 2, with random

Table 1. Experimental domain

Factor	Level −1	Level +1	Nominal
pH	2.0	3.0	2.5
Buffer concentration (mM)	25	100	62.5
β-Cyclodextrin concentration (mM)	10	20	15
Voltage (kV)	20	30	25

Table 2. Experimental design

Run	Voltage	pH	Buffer concentration	β-Cyclodextrin concentration
1	+1	+1	−1	+1
2	+1	+1	+1	+1
3	+1	+1	−1	−1
4	−1	+1	+1	−1
5	+1	−1	+1	−1
6	−1	−1	−1	+1
7	−1	+1	−1	−1
8	+1	−1	+1	+1
9	0	0	0	0
10	−1	+1	+1	+1
11	−1	−1	+1	+1
12	−1	−1	+1	−1
13	+1	−1	−1	+1
14	+1	−1	−1	−1
15	+1	+1	+1	−1
16	−1	−1	−1	−1
17	−1	+1	−1	+1

variation of the factors, were conducted. The response variables were determined from the obtained electropherograms, which were used to obtain the several graph options. Some of them are shown in Figures 2–4.

Figure 2 shows the Pareto chart of standardized effects for the resolution of ZPN enantiomers. The magnitude of each effect is represented by a column and the line that across the column indicates how large each

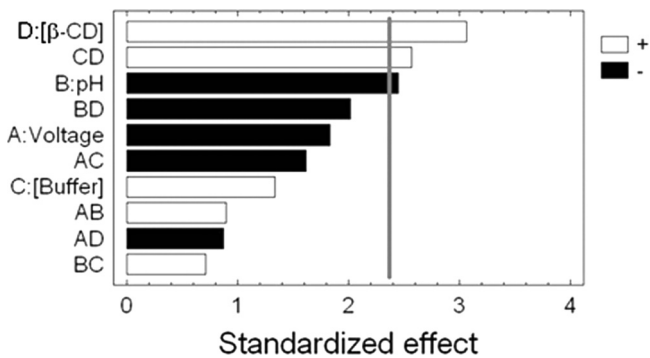
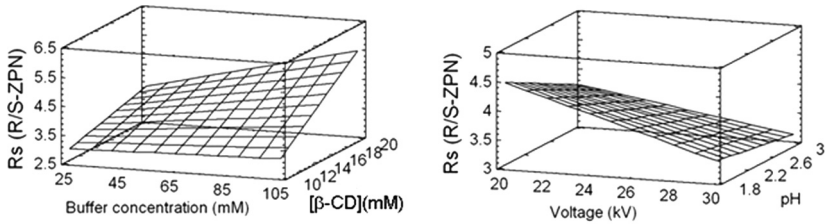


Figure 2. Standardized Pareto chart for Rs of ZPN enantiomers ($\alpha = 5\%$). CD, BD, AC, AB, AD and BC symbolize the interactions corresponding to the above mentioned letters.



(a) (b)

Figure 3. Response surface plot of the ZPN enantiomers resolution (Rs) as a function of (a) buffer ionic concentration and β-CD concentration, maintaining constant voltage (25 kV) and pH (2.0); (b) voltage and pH, maintaining constant buffer ionic concentration (62.5 mM) and β-CD (15 mM) concentration.

effect has to be. As can be seen in this graph, three factors affect, significantly ($\alpha = 5\%$), the ZPN enantiomers resolution, the β-cyclodextrin concentration, the interaction of the β-cyclodextrin and buffer concentration, and the pH.

Figure 3a is a three dimensional plot of the ZPN enantiomers resolution as a function of running buffer concentration and β-CD concentration. The surface plot allows the whole range of conditions to be explored, including combinations. As can be seen, both parameters, running buffer concentration and β-CD concentration, as well as their interaction, increase resolution between ZPN enantiomers. As depicted in Figure 3a, adequate resolution ($Rs = 3.8$) could be obtained under several conditions: maximum level of β-CD concentration (20 mM) with minimum level of buffer concentration (25 mM); medium level of β-CD

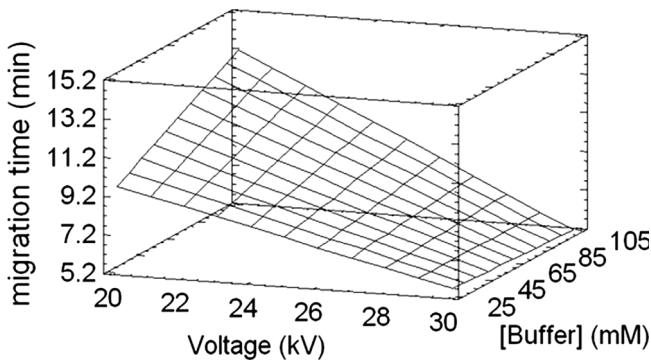


Figure 4. Response surface plot of the migration time as a function of buffer ionic concentration and voltage, maintaining constant β-CD concentration (15 mM) and pH (2.0).

concentration (15 mM) with medium level of buffer concentration (65 mM); and minimum level of β-CD concentration (12 mM) with maximum level of buffer concentration (100 mM).

Figure 3b is the response surface plot of the ZPN enantiomers resolution as a function of the voltage and the pH, maintaining constant running buffer concentration (62.5 mM) and β-CD (15 mM) concentration. As can be seen, the resolution decreases slightly when pH increases and obviously decreases more quickly when the voltage increase; in spite of that, the Pareto chart shows the voltage influence is less than 95%. In any case, the resolution obtained was always acceptable.

Figure 4 is the three dimensional plot of the *S*-ZPN migration time as a function of applied voltage and running buffer concentration. Obviously, the analysis time increases when the applied voltage decreases. On the other side, the influence of the buffer concentration in the migration time at high voltages was irrelevant.

The program allows us to know the ideal conditions to obtain the maximum resolution between ZPN enantiomers, the minimal analysis time, and to prevent ZPN and diazepam overlapping, assigning different levels of impact, from 1 to 5, to each variable. In order to obtain a short analysis time, an impact level of 5 was assigned to *S*-ZPN migration time. As the obtained resolution was good in all runs, an impact factor of 3 was assigned to this factor. A minimal impact level 1 was assigned to the resolution of ZPN enantiomers with diazepam.

As a result of the studies carried out, the ideal CE conditions obtained were: fused silica capillary of 48.5 cm length, 40 cm effective length, and 50 μm inner diameter; 60.2 mM phosphate buffer containing 20 mM β-CD and 1 M of urea, pH 2.0; voltage 30 kV. Figure 5 shows the electropherogram obtained with these conditions. As can be seen, the analysis time is lower than 7 minutes and the resolution between the ZPN enantiomers is 4.

Validation

Calibration lines were constructed by means of five-point analysis, and the resulting plots were lineal in the concentration range from 20 mg L^{-1} at least up to 600 mg L^{-1} of ZPN and its precursor. These analyses were carried out in triplicate, adding diazepam as an internal standard. The calibration graphs for all the compounds presented a good fit to a linear model between the peak relative areas and analyte relative concentrations. The regression coefficients were greater than 0.995 in all cases. The linearity of the calibration graphs were also tested by means of two different statistical tests: linearity and proportionality tests. For the former test, the linearity of the method was confirmed showing that

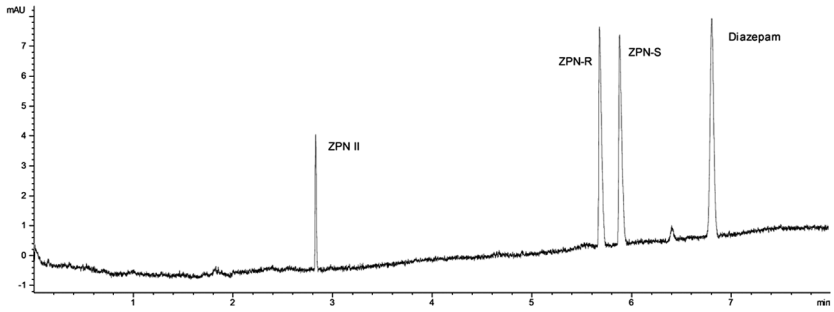


Figure 5. Typical electropherogram of a mixture of: (1) ZPN II; (2) (*R*)-ZPN; (3) (*S*)-ZPN; and (4) diazepam. CE conditions: fused silica capillary (48.5 cm length, 40 cm effective length, 50 μm inner diameter), phosphate buffer (60.2 mM, pH 2,0) containing 20 mM *β*-CD and 1 M urea; separation voltage 30 kV; capillary temperature 25°C; absorbance detection at 215 nm. Solutes concentration: 0.4 mg ml^{-1} of (*R*)-ZPN, (*S*)-ZPN and ZPN II and 0.2 mg mL^{-1} of diazepam.

the factor response RSD and the slope RSD values were lower than 5% and 2%, respectively. The values obtained from the Fisher test (analysis of variance, ANOVA) were always lower than the tabulated values ($F_{tab} = 2.958$; $\alpha = 0.05$). The calibration data are shown in Table 3.

In the proportionality test, it was demonstrated that the intercept was not statistically different from 0 and the Student's *t*-test values calculated were always lower than the tabulated values ($t_{tab} = 2.093$) for the same level of significance. This indicates the absence of systematic error, linearity thus being demonstrated.

The precision of the method was assessed by expressing the relative standard deviation of several repeated measurements (Table 4). Instrumental repeatability was estimated from six replicates at three concentrations (100, 300 and 500 mg L^{-1}) within the lineal interval, obtaining

Table 3. Calibration data

Range (20–600 mg L^{-1})	Zopiclone II	(*S*)-zopiclone	(*R*)-zopiclone	Specification
Correlation coefficient	0.998	0.998	0.995	≥0.99
Linearity test				
Response factor RSD	4.4	4.8	4.8	≤5%
Slope RSD	1.8	1.8	1.6	≤2%
ANOVA	2.18	1.38	1.20	$F_{exp} < F_{tab}$

$F_{tab} = 2.958$.

Table 4. Analytical characteristics of the electrophoretic method

	Specification	Zopiclone II	(*S*)–zopiclone	(*R*)–zopiclone
Injection repeatability (RSD%) (n = 6)	≤2%	0.4	1.8	1.7
Intermediate precision (RSD%) (n = 6)	≤5%	3.8	4.9	4.7
LOD mg l^{-1})		7.2	3.5	2.1
LOQ (mg l^{-1})		18.0	8.8	5.3

values (0.4% – 1.8%) below the acceptance criterion (≤2%). The estimation of repeatability was performed over a period of five hours. The compounds were stable and showed no significant difference in the peak area after this time. Intermediate precision was determined by comparing the results obtained from the analysis of freshly prepared samples on two separate days. The results, ranging between 3.8% and 4.9%, were also lower than the acceptance criterion (≤5%). Therefore, acceptable precision was obtained for all preparations.

The detection and quantification limits are shown in the aforementioned Table 4. These were determined by 10 repeated measures of the blank, followed by the preparation of calibration plots (peak height versus concentration) from 10 to 100 mg L^{-1} of each enantiomeric form. Detection and quantification limits were in the range of 2.1–7.2 and 5.3–18.0 mg L^{-1}, respectively.

Recovery experiments for all substances were performed in order to study the accuracy of the method. A mixture of known concentrations of each solute was prepared and analysed at low, medium, and high calibration ranges (100, 300 and 500 mg L^{-1}) by this method on the same day. All analyses were carried out in triplicate. The average recoveries obtained, which ranged between 97.6% and 100.7%, testify to the accuracy of method.

CE versus HPLC

Once the analytical method for determining the enantiomeric forms of ZPN was validated, a comparative study of the CE method versus the HPLC method was carried out. In this experiment, a sample of ZPN, doped with (*S*)-ZPN (*test sample*), was used to compare the results obtained by means of the two techniques (CE and HPLC). In the case of CE, the sample was prepared as previously described. In the HPLC analysis, the sample was dissolved in the mobile phase and the Mannaert's method,[9] previously mentioned, was carried out.

Table 5. Obtained values of the chromatographic and electrophoretic parameters

Parameter	(*R*)-ZPN	(*S*)-ZPN
HPLC		
Retention time (min.)	10.9	17.0
Number of plates	365	480
Symmetry	1.4	1.4
Peak width	3.99	4.05
Resolution	6.1	
Enantiomeric excess	46.1%	
CE		
Migration time (min.)	5.7	5.9
Number of plates	401111	154711
Symmetry	0.2	0.1
Peak width	0.04	0.06
Resolution	4.0	
Enantiomeric excess	48.7%	

Table 5 shows the obtained values of the chromatographic and electrophoretic parameters for all peaks of the ZPN mixture using both techniques (HPLC and CE). The enantiomeric excess value is included in the table.

Application to a Commercial Sample

The method has been applied to the control of a pharmaceutical formulation. A tablet of ZPN's commercial sample (7.5 mg of ZPN) was ground

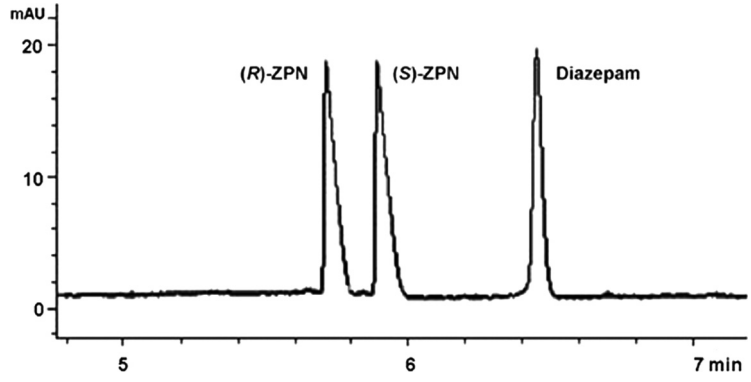


Figure 6. Electropherogram of a pharmaceutical formulation. Conditions were as in Fig 5. Peaks: (1) (*R*)-ZPN; (2) (*S*)-ZPN; (3) diazepam.

and 2 mg of diazepam as internal standard was added. The mixture was dissolved in acetonitrile, filtered, and transferred into a 10.0 mL volumetric flask, and raised up to volume with acetonitrile. The resulting filtered solution was placed in a CE vial to be analyzed. Figure 6 shows the electropherogram obtained. As can be seen, the commercial sample is a racemic mixture of both enantiomers. Since it has been established that the *S*-ZPN form is 50 times more active than its *R*-enantiomer, we venture to suggest that the use of pure drugs in the treatment of the sleep disorders would reduce the recommended dose, thereby decreasing the side effects while maintaining its benefits.

CONCLUSION

The factorial design used allowed us to optimize the CE method from a few experiences. The obtained results suggest that CE is an efficient method for the resolution and quantification of the ZPN enantiomers and its synthesis precursor in half the time of the HPLC method. The values of number of plates and width of peak are notably better than those obtained using HPLC. Furthermore, the CE method is cheaper and provided acceptable sensitivity, linearity, and repeatability. Therefore, this CE method will become an adequate alternative to chromatographic methods.

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