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New Micellar Electrokinetic Chromatographic Method for Analyzing Idebenone in Pediatric Formulations

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Abstract

A novel, simple and reliable method based on micellar electrokinetic chromatography with ultraviolet detection was developed to analyze idebenone in a pediatric formulation. Idebenone is a synthetic short chain benzoquinone that acts as an electron carrier in the mitochondrial electron transport chain facilitating the production of adenosine triphosphate. It can be found in two different redox states that differ in their physiological properties. Idebenone has been investigated as a treatment in several neurological disorders like Friedreich's ataxia, Leber's hereditary optic neuropathy, mitochondrial encephalomyopathies and senile dementia. Accordingly, a micellar electrokinetic chromatography was employed to discriminate both redox forms. The final optimized system was validated in terms of selectivity, linearity (r^2 0.992), limit of detection (0.5 μg/mL), limit of quantification (1.8 μg/mL), intra- and inter-day precision (RSD $\leq$ 2) and accuracy in terms of recovery studies (99.3–100.5%). Robustness was studied following a Plackett–Burman design. Finally, the validated system was applied to the analysis of idebenone in a pediatric formulation.

Introduction

Idebenone, 2-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-p-benzoquinone (Figure 1), is a synthetic short chain benzoquinone that acts as an electron carrier in the mitochondrial electron transport chain facilitating the production of adenosine triphosphate (1). In addition, it is an antioxidant that avoids lipid peroxidation and, thus, protects cell membranes and mitochondria from oxidative damage. Idebenone is the synthetic analog of the natural component of the mitochondria, Coenzyme Q10 (CoQ10) (2) sharing the same quinone ring, responsible of their action. However, idebenone has a modification of the composition and length of its side chain (1). These characteristics make idebenone a drug with a faster diffusion across biological membranes, and thus, it increases the energy supply and protects cells from peroxidative damage in a more efficient way with respect to CoQ10 (3). Idebenone could exist in two different oxidative states: the ubiquinol-derivative (reduced idebenone, idebenoneH_2) and the ubiquinone-derivative (oxidized idebenone). It was reported that both forms act via different mechanisms with dissimilar potency (4); therefore, it is important to assure the redox state of idebenone used for medical treatment.

In several neurological disorders, such as Friedreich's ataxia, Leber's hereditary optic neuropathy, mitochondrial encephalomyopathies and senile dementia, idebenone has been clinically investigated for treatment (1).

Several chromatographic methods have been developed to analyze idebenone in biological matrices (1, 5–8) using ultraviolet (UV), fluorescence, electrochemical and mass detection with different advantages. However, in the field of pharmaceutical analysis, there are

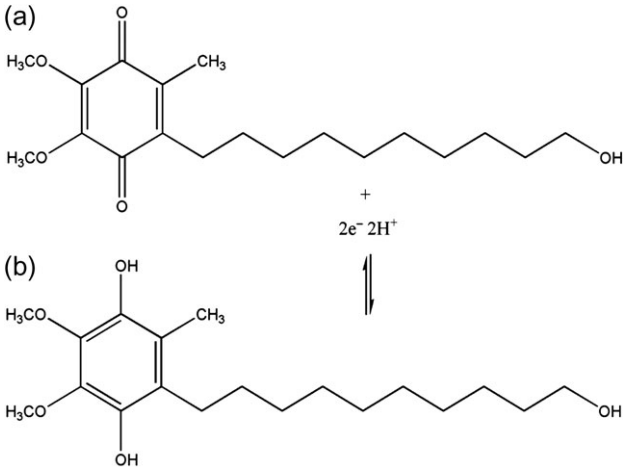

Figure 1. (a) Chemical structure of idebenone and (b) its reduced form.

a few analytical methods reporting the quantification of idebenone and its related compounds. Most of them were based on colorimetry, UV, gas chromatography, high-performance liquid chromatography (HPLC) and thin-layer chromatography and in some cases, methods were not targeted for the separation of any impurity (9–15).

A conventional HPLC-UV method able to evaluate degradation impurities as well as synthesis intermediates in bulk drug with high sensitivity and specificity useful in the quality control laboratory was recently developed (13). However, the reduced form of idebenone was not investigated.

Simplicity, accuracy and precision are key parameters in meeting the speed, quality standards and strict specifications required by the pharmaceutical industry. That is why it is extremely important to develop simple, affordable and high efficient analytical methodologies. In this sense, the miniaturization of chromatographic systems is a powerful tool to achieve these goals (16). Regarding simplicity and miniaturization, in the last decade, capillary electrophoresis (CE) with its different modes of operation has proven to provide great utility in the analysis of different types of compounds and has demonstrated to be an attractive alternative to traditional methodologies due to its high efficiency, short analysis time, wide range of analysis and the possibility of making changes in the electrolyte composition in order to separate a wide range of hydrophobic and hydrophilic compounds in complex matrices. Moreover, CE is considered an eco-friendly technique, which minimizes the use of organic solvents, it requires low amount of sample and it is also economic. (17).

Electrolyte additives have been used to modify the eletrophoretic mobility of analytes for better separation. In particular, the development of micellar electrokinetic chromatography (MEKC) in which micelles are added to the electrolyte as pseudo-stationary phase has greatly expanded the utility of the technique because it combines features of both liquid chromatography and CE techniques. The combination of both separation mechanisms results in a powerful tool that allows to separate complex mixtures of analytes as well as structurally similar analytes. The varying rates of partition between the complex analyte–micelle lead to excellent selectivity in separation (18, 19).

The aim of this work was to develop a simple analytical method able to discriminate both redox forms of idebenone using MECK. Moreover, this method was compared to an optimized HPLC method. Both methods were applied in the determination of idebenone in pediatric formulation. As far as we know, this is the first report for the analysis of idebenone in pharmaceuticals using CE.

Experimental

Reagents and chemicals

Idebenone (oxidized form) and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA), sodium borohydride (BH_4Na) was acquired from Riedel-de Haën and sodium tetraborate was from Carlo Erba (Rodano, MI, Italy). Tetrahydrofuran (THF), methanol and ethanol (HPLC grade) were supplied by Sintorgan (Buenos Aires, Argentina). Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, USA). All solutions were filtered through a 0.45 µm nylon membrane (Micron Separations, USA) before use.

Suspensions of idebenone consisted in dispersed idebenone in ORA PLUS®, with orange flavor, and tween or tocopheryl polyethylene glycol succinate as wetting agents.

Apparatus

CE analysis was carried out with a P/ACE™ MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA) and 32 Karat software was used to control the instrumental parameters. Uncoated fused silica capillary (MicroSolv Technology Corporation, Eatontown, NJ, USA) of 50 cm (40 cm length to the detector) x 75 µm intradermally (i.d.) was used in all CE separations.

HPLC analysis was performed using a Thermo Scientific HPLC (Waltham, MS, USA) equipped with a quaternary pump (P4000), a temperature control, a vacuum degasser (SCM 1000), a dual UV detector (UV2000), an automatic injector (AS3000) and Chrom Quest 5.0 software, which were used to control the instrumental parameters. Separations were performed using a Symmetry C-18 micro column (Waters, Milford, Massachusetts, USA; 75 mm × 4.6 mm i.d., 3.5 µm particle size).

Additional instrumentation including an Agilent 8452 diode array spectrophotometer (Santa Clara, California, USA) and an ultrasonic bath (Transsonic Digitals, ELMA, Kolpingstr) were used.

Procedure

Preparation of standard solution of idebenone

The standard solution of idebenone (1 mg/mL) was prepared in ethanol, protected from light and stored at −20°C. It was sonicated and brought to room temperature before using.

Preparation of reduced form of idebenone

The reduced form of idebenone (idebenoneH_2) was prepared from reduction of idebenone with sodium borohydride (BH_4Na). One milliliter of idebenone solution (1 mg/mL) in ethanol was added to 4 mL of BH_4Na (1.2 mg/mL) in water.

The mixture was allowed to stand protected from light for 5 minutes in an ice bath. Then the reduced form of idebenone standard solution was properly diluted and immediately injected (8, 20).

Sample preparation from pediatric formulation

An amount of 1.0 mL of idebenone suspension (50 mg/mL) was diluted to 50 mL with ethanol. The mixture was manually stirred, sonicated for 10 minutes and then centrifuged at 2000 *g* for 5 minutes. An amount of 1.0 mL of the supernatant was diluted to

25 mL with water with 6% of ethanol for the MEKC system or mobile phase for the HPLC system.

Operation conditions

Before the first use, the capillary was conditioned by rinsing at 30 psi with 0.5 mmol/mL KOH for 5 minutes, 0.1 mmol/mL KOH for 5 minutes, water for 10 minutes and finally with the separation electrolyte for 10 minutes. Sample vials storage and the separation were carried out at 25°C.

The samples were injected under 0.5 psi pressure for 5 seconds, and electrophoretic system was operated under positive polarity at 20 kV with a voltage ramp to 88 kV/min in 0.17 minutes.

Capillaries were rinsed before each sample with the separation electrolyte for 1 minute. A wavelength of 280 nm was selected to record the electropherograms. Dodecanone was selected as the micellar marker.

The HPLC system was adapted from the method developed by Artuch et al. (1) with slight modifications. The mobile phase of the HPLC method consisted in a mixture of methanol: water 80:20 (v/v), at a 1 mL/min flow rate. A wavelength of 280 nm was selected to record the chromatograms. Those conditions allowed a good resolution between both redox states, and the analysis was performed in a short time (<8 minutes).

Validation parameters

Validation was performed according to international guidelines (21, 22). The evaluated parameters were specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness.

The specificity of the method was studied by accelerated stress conditions (acid, alkaline, oxidation and light) and also examined by comparing electropherograms of excipient blanks of the pharmaceutical formulation. The following procedure was used for each stress condition:

Oxidation: 25 mL of a 3% v/v hydrogen peroxide (H_2O_2) solution was added to 25 mg of idebenone accurately weighed. Acidic: 25 mg of idebenone with 25 mL of 1 mmol/mL hydrochloric acid (HCl) solution were refluxed during 1 hour. Alkaline: 25 mg of idebenone with 25 mL of 0.05 mmol/mL sodium hydroxide (NaOH) solution. Light: 1 mg/mL of idebenone solution in ethanol was exposed to white light during 3 days.

Linearity was determined at five concentration levels of idebenone assayed by triplicate. Precision was evaluated for intra-day ($n = 6$) and inter-day assays ($n = 18$), and it was expressed as RSD for migration time and peak area at three different concentrations (80, 100 and 120% of the nominal value).

LOD and LOQ were calculated on the basis of the signal-to-noise evaluation. LOD was defined as the concentration giving a peak height three times the levels of the baseline noise. The LOQ was defined as the concentration giving a peak height 10 times the levels of the baseline noise.

Accuracy was calculated from recovery studies. Placebo samples prepared with all excipients contained in the pediatric formulation were spiked with idebenone at 80%, 100%, and 120% concentration levels (with respect to the nominal value). Preparations of each level were assayed by triplicate.

Robustness was studied following a Plackett–Burman (PB) design (23) and described in the supplementary data. Each variable was compared to a critical E statistic, which is calculated as the product between a tabulated t critical value ($P < 0.05$) and the standard deviation of each variable.

Results

Preparation of reduced form of idebenone

Figure 2 shows the UV spectra of idebenone and idebenoneH_2. Idebenone showed a maximum absorption at 280 nm. In its reduced form, the maximum shifted to 290 nm, there is a decrease in the absorption coefficient and its orange color disappeared. At 280 nm the absorption coefficient for idebenoneH_2 was 5 times lower than idebenone.

The reduction of idebenone was reversible, by adding an oxidant such as benzoquinone or hydrogen peroxide.

On the basis of these results, a detection wavelength of 280 nm was selected to record the idebenone chromatograms and electropherograms. A wavelength of 290 nm was also selected to record the electropherograms in order to enhance the idebenoneH_2 response

Separation of both oxidative states of idebenone

The optimized system consisted in a solution of SDS 50 mmol/L in sodium tetraborate 20 mmol/L with 3.5% of THF. The effect of adding THF to the MEKC-SDS system is showed in Figure 3A.

It was found that THF allows the resolution between both redox forms of idebenone, being 3.5% THF required for an appropriate resolution (Rs > 1.5). An increase in the THF concentration did not show a major impact on the resolution and extended migration times. The effect of the length and inner diameter of the capillary was studied. A capillary of 50 cm length and 75 µm i.d. was employed because good resolution and better detectability were obtained with suitable intensity of current (90 µA). The optimal voltage applied was found to be 20 kV in order to achieve the best resolution without excessive intensity of current. Higher voltages gave faster migration times but poorer resolution.

Sample introduction time was tested in the 1- to 10-second range and different pressures between 0.1 and 1.5 psi were applied, being 0.5 psi at 5 seconds the best condition for obtaining adequate peak symmetry, peak area and resolution of idebenone redox forms.

The impact of sodium tetraborate concentration on resolution was also investigated, maintaining 3.5% of THF. The use of 10 and 15 mmol/L sodium tetraborate concentration resulted in a lower resolution (Rs < 1). Higher concentrations of sodium tetraborate were not tested to avoid high currents and extended analysis times. A concentration of 20 mmol/L resulted to be optimal.

The percentage of ethanol in the injection was also tested from 5% to 30%. A decrease in the number of theoretical plates and deterioration in peak shape were observed when the proportion of

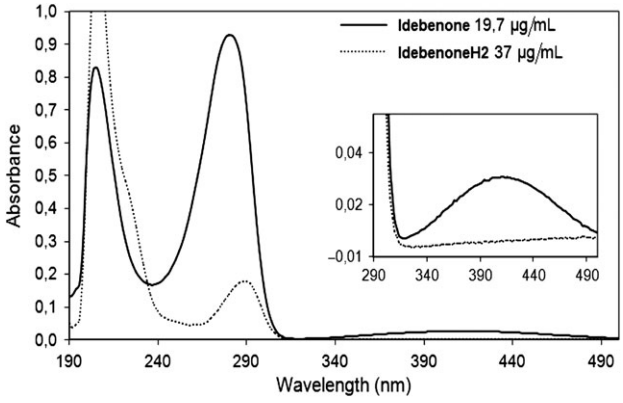

Figure 2. UV spectra of idebenone and idebenoneH_2.

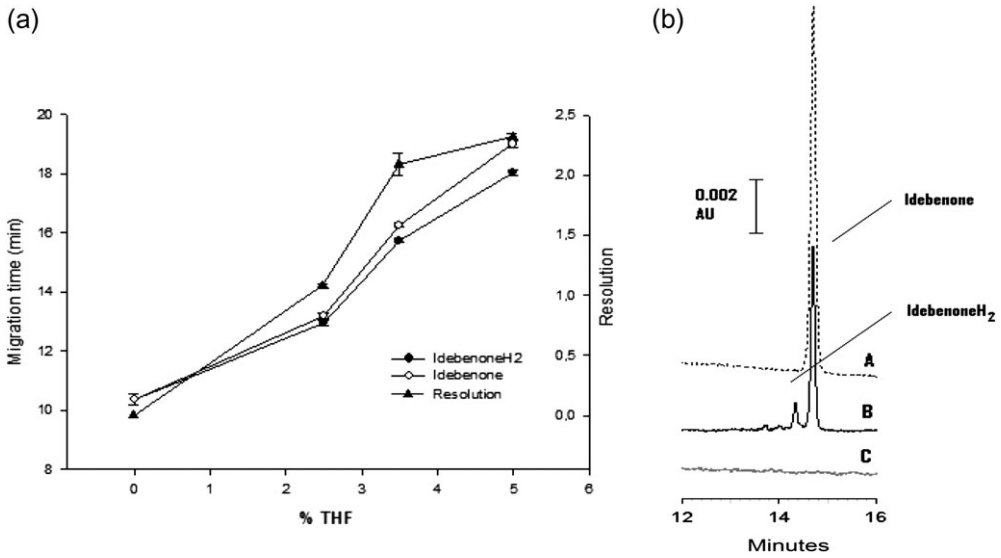

Figure 3. (a) Effect of the addition of THF on the migration time and resolution between idebenone and reduced idebenone (idebenoneH2). (b) Electropherogram employing the optimized system (20 mmol/L sodium tetraborate, 50 mmol/L SDS, 3.5% THF) of (A) Idebenone standard. (B) Standard mixture:idebenoneH_2 and idebenone. (C) Blank.

ethanol in the sample was increased. On the basis of this observation, 6% of ethanol in the injection was selected. The sample vials storage and separation were performed at 25°C, which allowed a good resolution and also protected the reduced form of idebenone (idebenoneH_2) from oxidation. At lower temperatures, broad peaks were obtained, and higher temperatures produced the oxidation of the reduced form of idebenone. The electropherogram of the separation of both oxidative states of idebenone using the optimized system is shown in Figure 3B.

MEKC developed method was compared with the traditional HPLC methods employed to quantitate idebenone. Despite both optimized systems showed acceptable chromatographic parameters, some differences were observed. The chromatographic parameters of resolution, asymmetry and number of theoretical plates from idebenone analysis were 2.25, 0.94 and 85080 for the MEKC method and 4.85, 1.34 and 4085 for the HPLC method.

The MEKC method presented a higher number of theoretical plates ~20 times higher than the HPLC method. The best peak shape of idebenone in terms of symmetry was achieved employing the MEKC method. Finally, in terms of resolution the HPLC presented an advantage compared to the MEKC method.

Validation of the analytical methods

Specificity

Idebenone showed a complete degradation when it was exposed to a strong alkaline condition (1 mmol/mL NaOH refluxed 1 hour). The alkaline stress test employing different NaOH solution concentration showed that idebenone is particularly susceptible to alkaline degradation. The alkaline stress test was performed with a 0.05 mmol/mL NaOH solution, which allowed a partial degradation of idebenone. Under acidic conditions (1 mmol/mL HCl), idebenone is partially degraded. Exposure to light stress resulted in a partial degradation of idebenone. Oxidation seems to not affect idebenone.

IdebenoneH_2 was oxidized to idebenone, when it was exposed to the stress conditions.

Figure 4A shows the electropherogram of idebenone under different degradation conditions. UV spectra corresponding to the main peak of acid, alkaline and light stress conditions compared to idebenone standard without degradation (STD) are also showed (Figure 4B). The peak purity was evaluated normalizing and comparing spectra from several peak sections.

Electropherograms of excipients blank of the pharmaceutical formulation did not show any peak under these conditions.

Precision, linearity, LOD, LOQ and accuracy

Table I shows the validation parameters of both, MEKC and HPLC, analytical methods for the determination of idebenone. Intra- and inter-day precision results were satisfactory at three different levels.

A comparison showed that both methods fulfill the proposed specifications. The results from the recovery assay are similar for both methods. However, the HPLC method showed a slightly better precision, as well as lower LOD and LOQ, and a better coefficient of correlation (r^2).

Robustness

The purpose of robustness is to evaluate the influence of small changes in the operating conditions on the responses of the method (24); therefore, the selected parameters were chosen because they are likely to be significant in practice.

Table II shows the effect and the critical statistic on different parameters of idebenone using the MEKC method. The critical statistic was higher than the value of the effect of the selected variables on different parameters, which confirmed the robustness of the analytical method.

Application: analysis of pediatric formulations

Determination of idebenone in two different suspensions (named 1 and 2) was performed under the experimental conditions described above (Figure 5) using an external standard quantification procedure.

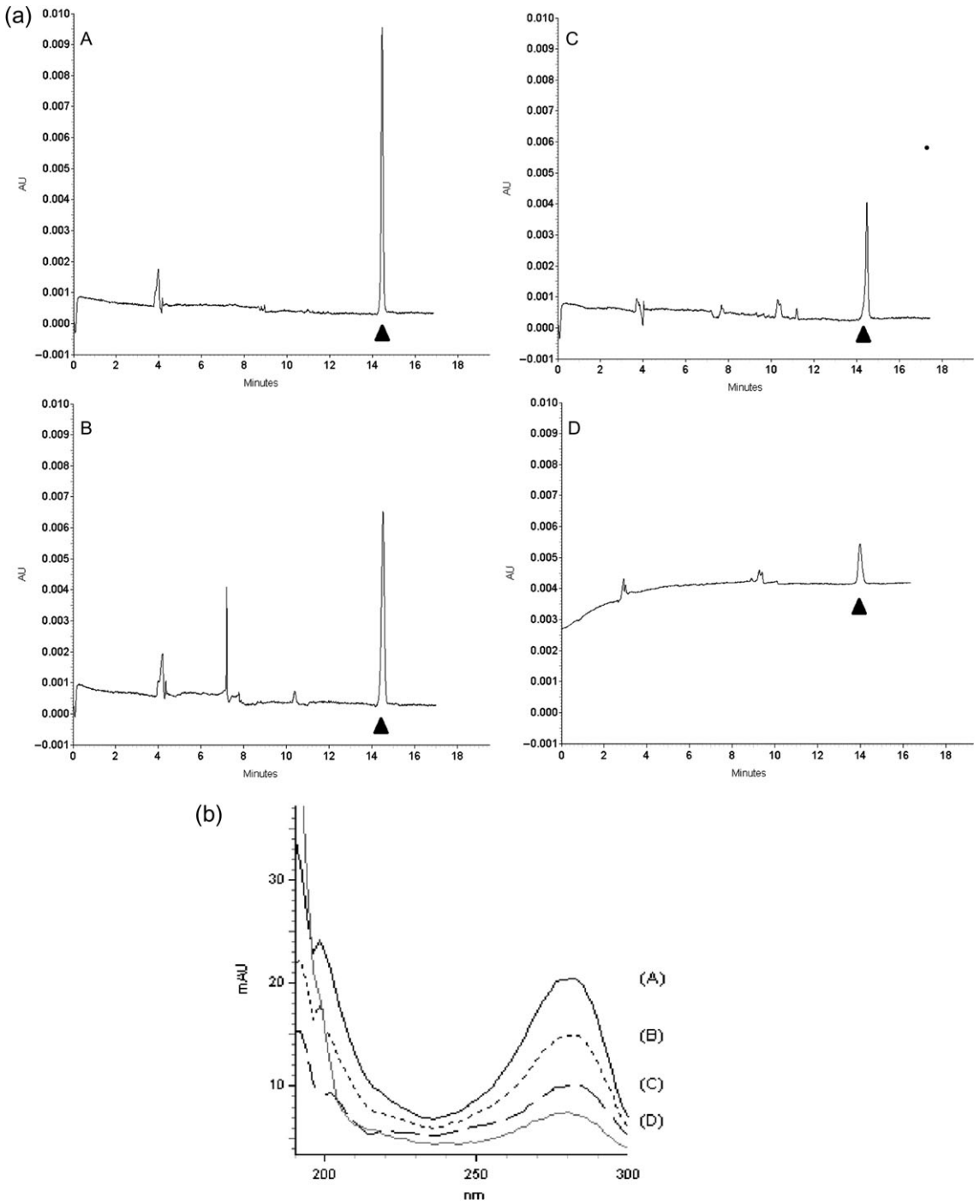

Figure 4. Idebenone under different degradation conditions analyzed by CE (a) and UV spectra corresponding to the main peak (b). (A) Idebenone standard▲, (B) idebenone acidic degradation, (C) idebenone alkaline degradation, (D) idebenone light degradation.

The results are in good agreement with the nominal values of the dosage form (50 mg/mL), and the reduced form of idebenone was not detected. The content of idebenone in Suspension 1 was 51.3 mg/mL and in Suspension 2 was 52.2 mg/mL using the MEKC method, whereas the content of idebenone in Suspension 1 was 50.0 mg/mL and in Suspension 2 was 53.7 mg/mL using the HPLC method. The RSD was <2 in both cases. These results showed a difference in the content of idebenone <1.4 % between both methods.

Discussion

In a previous work, our group developed a method for determination of CoQ10 based on CE using a double tensioactive microemulsion (25). Earlier, it has been demonstrated the impossibility of using a traditional MEKC system employing SDS as tensioactive due to the high hydrophobicity of CoQ10 (XlogP = 19.4) (26). Considering that idebenone (XlogP = 4.3) (26) is a neutral molecule but much less hydrophobic than CoQ10, it seems consistent to use

Table I. Comparison of MECK and HPLC Validation Parameters for Idebenone Analysis

Parameter	MECK	HPLC
Idebenone		
Linear range	1.8-60.0 μg mL^{-1}	1,0-60.0 μg mL^{-1}
r^2	0.992	0.999
LOD	0.5 μg mL^{-1}	0.3 μg mL^{-1}
LOQ	1.8 μg mL^{-1}	1.0 μg mL^{-1}
Precision at different levels (RSD)		
Intra-day ($n = 6$)		
80% Migration/retention time	0.33	0.11
80% Peak area	1.26	0.63
100% Migration/retention time	0.76	0.25
100% Peak area	1.05	0.60
120% Migration/retention time	1.85	0.21
120% Peak area	1.62	0.70
Precision at different levels (RSD)		
Inter-day ($n = 18$)		
80% Migration/retention time	2.01	0.34
80% Peak area	1.94	0.84
100% Migration/retention time	1.94	0.81
100% Peak area	1.91	0.98
120% Migration/retention time	1.91	0.28
120% Peak area	1.96	0.93
Accuracy		
Spiked levels (RSD in brackets)		
80%	100.5 (1.80)	101.8 (0.83)
100%	99.9 (1.83)	100.6 (1.61)
120%	99.3 (1.85)	101.6 (1.17)
$IdebenoneH_2$		
LOD	2.75 μg mL^{-1}	1.65 μg mL^{-1}
LOQ	9.90 μg mL^{-1}	5.50 μg mL^{-1}

Table II. Robustness of CE Method: Effect and Critical Statistic on Different Parameters Assayed

Parameter	*N*	*k'*	*T*	*R*	Content of idebenone
Voltage	−0.50	0.26	−0.02	0.12	1.33
Temperature	−10.50	0.12	0.00	0.04	−0.08
Ethanol in the injection	9.00	0.03	-0.01	0.06	−1.90
THF concentration	-8.50	0.00	0.03	0.05	−1.01
Borate concentration	−10.00	0.29	−0.04	0.04	1.14
Wavelength	1.00	−0.10	0.04	0.01	−1.12
Time of rinse	−1.00	0.01	0.00	0.02	−0.30
Critical statistic	34.18	0.76	0.13	0.27	5.38

N, theoretical plates; *k'*, retention factor; *T*, tailing; *R*, resolution.

an MEKC with SDS to develop a method for idebenone determination by CE.

Therefore, the separation between the oxidized and reduced forms of idebenone was investigated at first using an MEKC-SDS system consisting of 50 mmol/L SDS and 20 mmol/L sodium tetraborate as the background electrolyte. In these conditions, oxidized and reduced forms could not be separated. Moreover, migration time of idebenone was closed or overlapped with the micelle migration time.

Regarding the addition of organic solvents like methanol and THF to tensioactives, it is known that incorporation of organic solvents modulates separation of analytes and helps in their resolution because the organic solvents enlarge the elution window allowing better

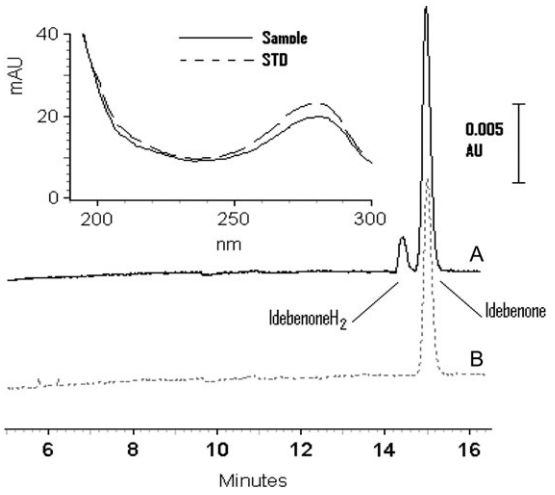

Figure 5. Analysis of a pediatric suspension of idebenone by CE. The UV spectrum of each peak is also showed. (A) Standard of idebenone and $idebenoneH_2$. (B) Pediatric suspension.

separation (25). On the basis of those results, THF was selected as organic modifier to increase the resolution and enlarge the elution window, which led to the separation of both oxidative states of idebenone.

In terms of idebenone quantification, some authors preferred to quantify idebenone in biological or pharmaceutical matrices using chromatographic methods with internal standards (7, 9). Traditionally, internal standard quantification is used to overcome the decrease of accuracy and precision of the method, and the choice of internal standard should be extremely careful. However, if the procedure including the sample preparation is simple and allows a high recovery, the use of internal standard is not needed (27). In this work, the addition of an internal standard was unnecessary because a high recovery is achieved, making it a simpler method.

Both methods presented suitable chromatographic parameters. The HPLC system showed a better precision and a shorter running time than the MEKC method. However, the principal advantage of the MEKC method is the low volume of electrolyte used (10 mL), compared to the volume of mobile phase used in the micro-HPLC system (300 mL) and the low volume of organic solvent consumed (350 μL vs. 240 mL), making it environmentally friendly and also more economic. The MEKC system also presented an excellent number of theoretical plates.

Conclusion

A novel micellar electrokinetic chromatographic method has been developed for the analysis of idebenone and its performance was compared to an HPLC method. Finally, the MEKC method was successfully applied to the analysis of idebenone in a novel pediatric formulation without significant difference compared to the HPLC method. In conclusion, the developed method is suitable to be used for quality control of idebenone in pediatric formulations as well as stability indicating studies.

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online.

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