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Determination of the dissociation constants of ropinirole and some impurities and their quantification using capillary zone electrophoresis

Pavel Coufal[a,*], Karel Štulík[a], Henk A. Claessens[b], Martin J. Hardy[c], Michael Webb[c]

[a]*Department of Analytical Chemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic*
[b]*Laboratory for Instrumental Analysis, Eindhoven Technical University, P.O. Box 513, 5600 MB Eindhoven, The Netherlands*
[c]*Analytical Sciences, SmithKline Beecham Pharmaceuticals, Third Avenue, The Pinnacles, Harlow, Essex CM19 5AW, UK*

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Abstract

Ropinirole, 4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one, is a potent anti-Parkinson's disease drug developed by SmithKline Beecham Pharmaceuticals. Capillary zone electrophoresis (CZE) was used for the determination of the dissociation constants of ropinirole and five structurally related impurities, potentially formed during its synthesis and for separation and quantification of these substances. The dissociation constants obtained from the CZE measurements were confirmed by UV spectrophotometry for some of the test compounds, obtaining a good agreement between the values. Careful optimization of the running buffer composition permitted base-line resolution of the six compounds in a borate buffer containing acetonitrile and magnesium sulfate (a 100 m*M* borate buffer containing 30 m*M* $MgSO_4$ and 20 vol.% of acetonitrile). It was shown that CZE can determine the level of these impurities, down to a level of 0.05% of the main component within 15 min.

Keywords: Dissociation constants; Ropinirole

1. Introduction

It has been demonstrated that CZE is a very powerful separation technique in analyses of a wide range of compounds [1–8] that can also be successfully applied for the determination of dissociation constants of weak acids and bases as demonstrated in the literature [9–14]. This method offers several advantages over potentiometric and spectroscopic techniques, among which a negligible consumption of the analyte seems to be most characteristic [9,11]. The CZE technique can also be modified for determination of the dissociation constants of water-insoluble or sparingly soluble compounds [15].

Ropinirole, 4-[2-(dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one, is a potent anti-Parkinson's disease drug which has been developed by SmithKline Beecham Pharmaceuticals. This compound of interest may be accompanied by five structurally related impurities originating from the synthesis procedure. Camilleri [16] has shown that

*Corresponding author.

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ropinirole could be separated from some related metabolites using CZE.

This work deals with a CZE determination of the dissociation constants of ropinirole and its five related impurities from the synthetic procedure; the dissociation constants of some of the compounds have been confirmed by UV spectrophotometry. The technique was further used for quantitative determination of ropinirole and its most common impurities.

2. Theory

When a weak base B exhibits the protonation equilibrium

$$BH^+ + H_2O \Leftrightarrow B + H_3O^+ \quad (1)$$

characterized by the apparent acidic dissociation constant

$$K'_a = \frac{[B] \cdot [H_3O^+]}{[BH^+]}, \quad (2)$$

the effective electrophoretic mobility of the analyte B depends sigmoidally on the separation buffer pH according to Eq. (3) [11,14]

$$m_{eff,B} = m_{BH^+} \cdot \frac{1}{1 + 10^{(pH - pKa')}} \quad (3)$$

where $m_{eff,B}$ is the effective electrophoretic mobility of the analyte B under the given pH obtained from an electropherogram and m_{BH^+} is the electrophoretic mobility of the ion BH^+, i.e., the effective electrophoretic mobility of the analyte B at a pH at which all the molecules of B are totally protonated.

The effective electrophoretic mobilities of an investigated analyte, measured by CZE at various pH values of the separation buffer and plotted against the pH, can be fitted with the sigmoidal model represented by Eq. (3) using non-linear regression and the acidic dissociation constant of the analyte is then obtained as one of the regression parameters. The non-linear regression characterized by the sigmoidal model should be preferred to a linear regression as the least biased dissociation constants are obtained [14].

The apparent acidic dissociation constants of bases obtained from the sigmoidal model must be corrected for the buffer ionic strength to obtain the thermodynamic values [13,14]

$$pK_a = pK'_a - \frac{0.5085 \cdot z^2 \cdot \sqrt{I}}{1 + 1.6404 \cdot \sqrt{I}} \quad (4)$$

where I is the buffer ionic strength and z is the buffer ion valence.

Analogously, the dissociation constants can be obtained spectrophotometrically [13,17], using the sigmoidal model represented by Eq. (5)

$$A = A_0 + A_{BH^+} \cdot \frac{1}{1 + 10^{(pH - pK_a')}} \quad (5)$$

where A is the absorbance of the actual solution, A_0 is the absorbance of the matrix and A_{BH^+} is the absorbance of the solution where only the totally protonated form of the analyte B is present.

3. Experimental

3.1. Chemicals

The buffers employed to measure the sigmoidal curves both in CZE and UV-spectrophotometry are listed in Table 1. Thiourea (Lachema, Czech Republic) in a 1 m*M* concentration was used as the electroosmotic flow marker. Cyclohexylaminopropanesulfonic acid (CAPS), boric acid, magnesium sulfate, sodium hydroxide and acetonitrile were purchased from Merck (Darmstadt, Germany). Ropinirole and its five related impurities (for structures and numerical labels see Fig. 1) were provided by SmithKline Beecham Pharmaceuticals (Tonbridge, UK). The water used for preparation of all the solutions was purified with a Milli-Q Water Purification System (Millipore Corp., USA). All the chemicals were of the ‘pro analysis’ purity and were used as received.

3.2. Apparatus

A Prince version 1 programmable injector for capillary electrophoresis (Lauerlabs, Emmen, The Netherlands) was used to perform the CZE experiments for determination of the dissociation constants. The oven integrated in this injector was utilized to

Table 1
Buffers used in the measurements of the sigmoidal curves

pH	Components[a]	Concentration (m*M*)
2.20	Sodium dihydrogen phosphate dihydrate	10
	Phosphoric acid	10
3.70	Sodium formate	10
	Formic acid	10
4.55	Sodium acetate	10
	Acetic acid	10
6.36	2-(*N*-morpholino)ethanesulfonic acid (MES)	20
	Sodium hydroxide	10
8.24	Tris(hydroxymethyl)aminomethane (Tris)	20
	Acetic acid	10
9.05	Sodium tetraborate (borax)	10
	Boric acid	10
9.67	2-Aminosuccinamic acid (asparagine)	20
	Sodium hydroxide	15
10.05	Cyclohexylaminopropanesulfonic acid (CAPS)	20
	Sodium hydroxide	4
10.72	Cyclohexylaminopropanesulfonic acid (CAPS)	20
	Sodium hydroxide	10
11.42	Triethylamine	20
	Hydrochloric acid	2

[a] CAPS and MES were purchased from Sigma (St. Louis, MO, USA) and all the other chemicals were supplied by Merck (Darmstadt, Germany).

thermostat the capillary columns at 30°C. The Prince was accompanied with a dc power supply (±35 kV, HCN 140-35000, FUG Electronik GmbH, Germany), controlled by the programmable injector, and an on-column UV absorbance detector (Unicam Analytical Systems, Cambridge, UK) operated at 250 nm. Platinum wires were used to connect the injection unit to the positive electrode and the buffer reservoir on the detector side to the grounded electrode. A Tulip AT Compact 3 computer ('s-Hertogenbosch, The Netherlands) with a home-made Multilab-TS interface and the Caesar program (B-Wise Software, Geleen, The Netherlands) were employed for data acquisition and handling.

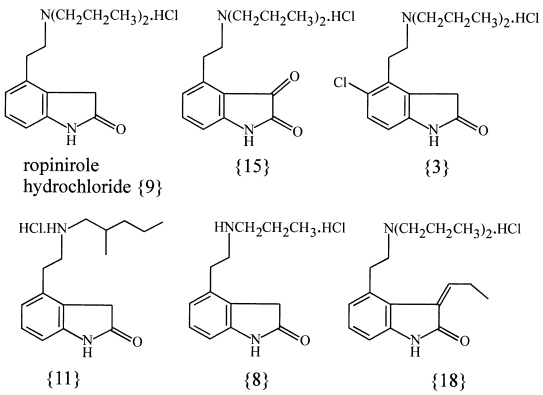

Fig. 1. Structures and numerical labels of ropinirole and its impurities.

An open, polyimide-clad fused silica capillary (J&W Scientific, Folsom, USA) of 50 μm I.D., 375 μm O.D., a 40-cm inlet-to-detector length and of 55 cm total length was used for the CZE experiments. The separation voltage applied in the CZE experiments was 20 or 30 kV with a voltage ramp-up of 6 kV/s.

An ultraviolet spectrophotometer SP 800 A (Unicam Analytical Systems, UK) using 0.5-cm quartz cuvettes was employed to measure the spectra of the investigated analytes.

A P/ACE System 5500 with a diode array detector (Beckman Instruments, Palo Alto, CA, USA) was used to perform the CZE separation and quantification experiments. A 47-cm long, open, polyimide-clad fused-silica separation capillary (J&W Scientific, Folsom, USA) 50 μm I.D., 375 μm O.D., was used, with an inlet-to-detector distance of 40 cm. The

separation voltage applied was 30 kV with a voltage ramp-up of 2.5 kV/s. The typical operating currents with the optimum separation buffers were between 50 and 60 μA. The diode array detector was operated at a constant wavelength of 254 or 200 nm. All the quantification experiments were performed at the wavelength of 254 nm. Samples were injected using a hydrodynamic injection method with injection programmes 35 mbar for 2 s (ca. 2.5 nl) or 35 mbar for 10 s (ca. 11 nl).

4. Results and Discussion

4.1. Dissociation constants

The migration times of all the investigated compounds were measured in CZE experiments employing buffers of different pH values listed in Table 1. The effective electrophoretic mobilities were then calculated from the migration times corrected for the delay caused by ramping of the applied voltage [20], using the equation

$$m_{\mathrm{eff},i} = \frac{l_\mathrm{d} \cdot l_\mathrm{t}}{U} \cdot \left(\frac{1}{t_{\mathrm{mig},i} - \frac{U}{2R}} - \frac{1}{t_\mathrm{eof} - \frac{U}{2R}} \right) \qquad (6)$$

where l_d is the length of the capillary to the detector window, l_t is the total length of the capillary, $t_{\mathrm{mig},i}$ is the migration time of the ith analyte, t_eof is the migration time of thiourea, U is the voltage applied and R is the voltage ramp-up [V/s]. The effective electrophoretic mobilities obtained from these experiments are summarized in Fig. 2. All the points are the arithmetic means of at least three measurements with R.S.D. values lower than 5%.

The experimental points in Fig. 2 were subjected to non-linear regression (Eq. (3)) using the Origin software and the sigmoidal curves obtained were fitted to the experimental effective electrophoretic mobilities. The regression parameters of the sigmoidal curves obtained are given in Table 2.

To obtain the thermodynamic dissociation constants, the apparent constants were corrected using Eq. (4). Most running buffers consisted of half-dissociated monoprotic acids (the concentration of monovalent acid anions of 10 mM). Therefore, the

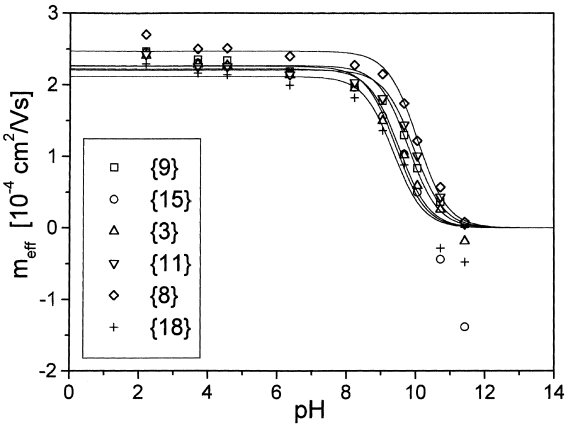

Fig. 2. Experimental effective electrophoretic mobilities (points) and fitted sigmoidal curves as the function of the running buffer pH.

buffer ionic strength was $I = 0.5 \cdot 0.01 \cdot 1^2 = 0.005$ and the negative correction factor from Eq. (4) equalled 0.03 for the buffer ion valence $z = 1$. The calculated correction factor of 0.03 was always within the 95% confidence limits of the apparent dissociation constants in Table 2, i.e., the error of determination of the dissociation constants in our experiments was higher then the difference between the apparent and thermodynamic dissociation constants calculated.

The compounds {8} and {11} exhibited higher acidic dissociation constants than the other investigated analytes as these compounds are secondary amines. This agrees well with the literature [18]. The compounds {3}, {15} and {18} exhibit negative effective electrophoretic mobilities at the highest pH value investigated. This can be explained by tautomeric rearrangement at the structure element –NH–CO–. The double bond may shift between the nitrogen and carbon atoms, producing –OH group (located on the carbon atom) that can dissociate at high pH values. The dissociation of the hydroxyl group may be facilitated by the neighbouring substituents.

Apparently, Eq. (3) could only describe the first protonation equilibrium for the amine group and did not include the second protonation equilibrium for the hydroxyl group observed through the negative effective electrophoretic mobilities of the compounds {3}, {15} and {18}. An attempt was made to fit the experimental electrophoretic mobilities of the com-

Table 2
Regression parameters of the sigmoidal models in CZE, correlation coefficients between the experimental data and the sigmoidal curves in CZE and comparison of the apparent acidic dissociation constants obtained from CZE and UV spectrophotometry with the 95% confidence limits in parentheses

Compound[a]	pK_a' from CZE	m_{BH^+}	r	pK_a' from UV
{9}	9.79 (0.07)	2.27 (0.06)	0.991	9.48 (0.05)
{15}	9.43 (0.32)	2.26 (0.25)	0.955	9.54 (0.08)
{3}	9.52 (0.09)	2.22 (0.07)	0.989	—
{11}	9.95 (0.07)	2.21 (0.05)	0.991	10.20 (0.07)
{8}	10.06 (0.06)	2.47 (0.05)	0.992	—
{18}	9.36 (0.18)	2.12 (0.12)	0.986	—

[a] For structures see Fig.1.

pounds {3}, {15} and {18} with Eq. (7) including both of the described protonation equilibria

$$m_{\mathrm{eff,B}} = m_{\mathrm{BH}^+} \cdot \frac{1}{1 + 10^{(\mathrm{pH} - \mathrm{p}K_{\mathrm{a1}}')}} + m_{\mathrm{B}^-} \cdot \frac{1}{1 + 10^{(\mathrm{p}K_{\mathrm{a2}}' - \mathrm{pH})}} \quad (7)$$

where m_{BH^+} ($m_{BH^+} > 0$) and m_{B^-} ($m_{B^-} < 0$) are the electrophoretic mobilities of ions BH^+ and B^-, respectively, and $K_{a,1}'$ and $K_{a,2}'$ are the apparent acidic dissociation constants characterising the protonation equilibria for the amine and hydroxyl groups, respectively. However, the fit using Eq. (7) was not successful because of the lack of experimental data for the high pH values. The instability of the electroosmotic flow and decomposition of some of the investigated compounds at pH values above 12 made it impossible to obtain reliable experimental effective electrophoretic mobilities of the investigated compounds in this pH region. Based on this fact, Eq. (3) was applied to fit the experimental effective electrophoretic mobilities of all the investigated compounds, describing only the acid-base equilibrium of the amine groups.

The dissociation constants of compounds {9}, {11} and {15} were also determined spectrophotometrically. The experimental absorbances at wavelengths of 224 nm for {9}, 225 nm for {11} and 265 nm for {15} were plotted against the buffer pH and then these points were subjected to non-linear regression using the sigmoidal model represented by Eq. (5). This simple model is only valid in cases in which the absorbance of the non-protonated species is negligible, however, this model fitted our experimental data with correlation coefficients higher than 0.998. The apparent acidic dissociation constants from UV spectrophotometry are also summarized in Table 2, demonstrating good agreement between the dissociation constants determined by CZE and spectrophotometry. In the spectrophotometric experiments, the dissociation of the hydroxyl group was observed for compound {15}, with decomposition at high pH values (pH 13–14).

4.2. Separation

The largest differences in the effective electrophoretic mobilities of individual compounds can be expected at pH values close to their dissociation constants. Therefore, the following running buffers were selected and their compositions were optimized.

A CAPS buffer with pH 10.2 and borate buffers with pH 9.5, 8.7 and 8.5 at concentrations from 20 to 100 m*M*, containing magnesium sulfate at concentrations from 1 to 40 m*M*, with 15 or 20 vol.% of acetonitrile, were investigated, on the basis of our previous experience. The buffer pH was adjusted after adding magnesium sulfate and before adding acetonitrile, using 50 and 0.5 m*M* sodium hydroxide solutions. Acetonitrile was added to improve the solubility of the test compounds and to slow down the electroosmotic flow. Magnesium sulfate was used in these buffers to slow down the electroosmotic flow and to reduce adsorption of the analytes on the capillary surface through an electrostatic interaction of the magnesium cations with the negatively charged capillary wall.

The best CZE separations of the test substances

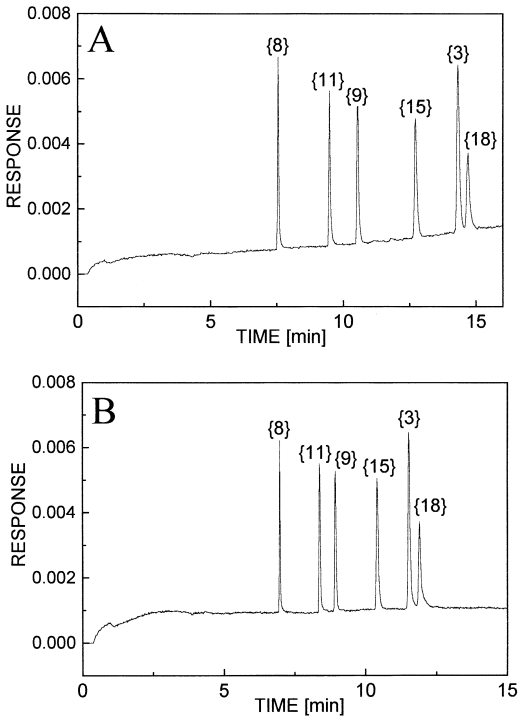

Fig. 3. Electropherograms of the analytes in a 100 m*M* borate buffer containing 30 m*M* $MgSO_4$ (pH 8.70) with 20 vol.% of acetonitrile (A) and a 100 m*M* borate buffer containing 40 m*M* $MgSO_4$ (pH 8.70) with 15 vol.% of acetonitrile (B). Aqueous sample, cca. 0.1 mg/ml of each compound: injection, 35 mbar for 2 s (ca. 2.3 nl); instrument, P/ACE System 5500; capillary, 50 μm×47 cm; effective length, 40 cm; separation, 30 kV, 51 μA (A) and 57 μA (B); detector, 254 nm.

were attained with two running buffers, namely, a 100 m*M* borate buffer containing 40 m*M* magnesium sulfate, pH 8.7, with 15 vol.% acetonitrile and a 100 m*M* borate buffer containing 30 m*M* magnesium sulfate, pH= 8.7, with 20 vol.% acetonitrile. Using these running buffers, the electropherograms given in Fig. 3 were obtained and all the test drugs were base-line separated within 15 min.

4.3. Quantification

The 100 m*M* borate buffer containing 30 m*M* $MgSO_4$ and 20 vol.% acetonitrile, pH 8.7, was selected to carry out the quantification experiments, as this running buffer generated a lower electrical current. To confirm linearity of the detector response to the concentration of the test substances, calibration curves (peak area versus concentration) were measured with ropinirole in a low concentration range from 0 to 25 μg/ml and a high concentration range from 0 to 2500 μg/ml and with the ropinirole impurities in the low concentration range from 0 to 25 μg/ml. The experimental data were subjected to linear regression obtaining the results that are summarized in Table 3.

All the intercepts of the calibration curves in Table 3 were found to be insignificantly different from zero at a significance level of $\alpha = 0.05$ using the *t*-test for intercepts ($t = a/sd_a$). A good agreement between the experimental points and the calibration curves was

Table 3
Linear regression parameters of the calibration curves (peak area = $a + b \cdot$conc. (μg/ml)) for ropinirole and its impurities, with the standard deviations in the parentheses[a]

Compound[b]	*a* (S.D.)	*b* (S.D.)	*r*	LOD (μg/ml)
{8}	−0.008 (0.008)	0.017 (0.000)	0.998	0.7
{11}	−0.004 (0.005)	0.009 (0.000)	0.998	0.6
{9}	−0.014 (0.012)	0.014 (0.001)	0.996[c]	1.1
	−0.280 (0.522)	0.013 (0.000)	0.998[d]	
{15}	0.011 (0.009)	0.017 (0.001)	0.998	0.9
{3}	0.007 (0.008)	0.025 (0.000)	0.999	0.8
{18}	0.002 (0.020)	0.027 (0.001)	0.996	1.0

[a] *a*, intercept; *b*, slope; *r*, correlation coefficient; LOD limit of detection.
[b] For structures see Fig.1.
[c] For the low concentration range from 0 to 25 μg/ml.
[d] For the high concentration range from 0 to 2500 μg/ml.

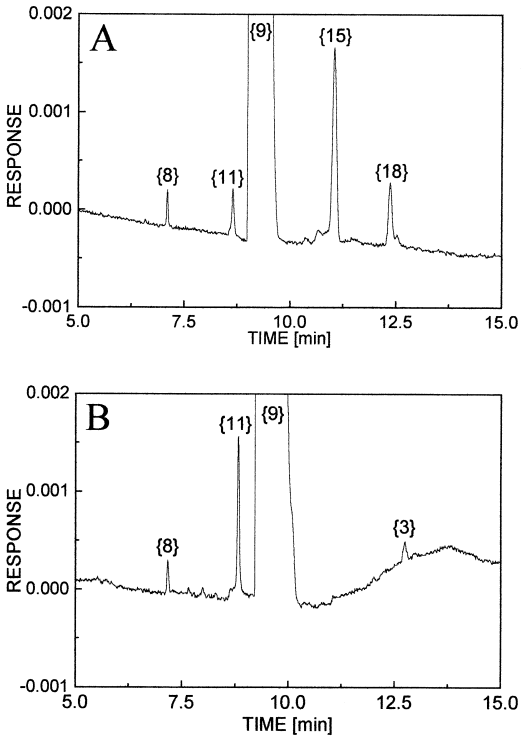

Fig. 4. Electropherograms of the No. 1 (A) and No. 2 (B) test batches of ropinirole in a 100 m*M* borate buffer containing 30 m*M* $MgSO_4$ (pH 8.70) with 20 vol.% of acetonitrile. Aqueous sample, 2 mg/ml; injection, 35 mbar for 10 s (ca. 11.4 nl); instrument, P/ACE System 5500; capillary, 50 μm×47 cm; effective length, 40 cm; separation, 30 kV, 50 μA; detector, 254 nm.

observed as follows from the correlation coefficients. In the worst case of the correlation coefficient value (i.e., $r=0.996$) observed for compounds {9} and {18}, the experimental point variation was explained from 99.2% (i.e., $100\cdot r^2$) by the calibration curve variation. The limits of detection were determined as three times root mean squared baseline noise [19].

The same borate buffer was also used to analyze the impurity profile of two real samples of ropinirole from its synthetic impurities; Fig. 4. As the slopes of the calibration curves, representing the CZE sensitivity for the individual analytes, were comparable and the level of impurities was very low, an internal normalization evaluation method was used to quantify the percentage of impurities in the ropinirole batches. The mean values of the area percent, the relative standard deviations and the impurity concentrations in the injected solution are summarized in Table 4. The highest impurity level determined, was 0.88% with relative standard deviation of 2.3%. Employing conventional HPLC, the following impurity concentrations were determined in the first ropinirole batch using the internal normalization evaluation method at the wavelength of 254 mn [21]: 0.1% of {8}, 0.2% of {11}, 1.1% of {15} and 0.3% of {18}; {3} was not detected. There was thus a satisfactory agreement between the CZE and HPLC data. The small differences between the two techniques may be due to the use of the internal normalization

Table 4
The impurity profile of the real samples No. 1 and No. 2 of ropinirole using the internal normalization evaluation method

Compound[a]	Mean of area (%)[b]		R.S.D. (%)		Concentration[c] (μg/ml)	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
{8}	0.09	0.07	4.6	6.8	1.8	1.4
{11}	0.12	0.23	2.9	3.2	2.4	4.6
{9}	98.69	99.65	0.02	0.05	2000	2000
{15}	0.88	nd	2.3	nd	17.6	nd
{3}	nd[d]	0.05	nd	19.7	nd	1.0
{18}	0.22	nd	3.1	nd	4.4	nd

[a] For structures see Fig.1.
[b] Nine repeated runs, $n=9$, for sample No. 1; nine repeated runs, $n=9$, for sample No. 2.
[c] The analyte concentration in the injected solution.
[d] nd, not detected.

evaluation methods, which is very often applied in impurity profiles of pharmaceutical compounds, and due to the dependence of the peak area on the zone migration velocity in CZE. The impurity concentration in the injected solution calculated from the mean of area per cent were higher than or comparable to the limits of detection determined as three times root mean squared baseline noise.

5. Conclusion

Acidic dissociation constants of ropinirole and its five common impurities were determined. CZE was demonstrated as a suitable technique for the determination of the dissociation constants of weak bases. The method was found to consume negligible amounts of compounds in comparison to UV spectrophotometric technique. A good agreement between the dissociation constants obtained by the two methods was found.

CZE has also been demonstrated as a powerful separation technique for structurally similar compounds. However, a careful optimization of the running buffer composition was necessary to obtain a base-line resolution of all the compounds. The work presented here underlines the importance of application of CZE separations of pharmaceutical compounds. CZE provides a very rapid impurity profile method for ropinirole batches with an analysis time not exceeding 15 min. It has been demonstrated that impurity levels lower than 0.05% can be determined using CZE within a very short analysis time, with relative standard deviations below 10%. A satisfactory agreement has been found between the impurity profiles determined by CZE and HPLC.

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