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Development and validation of a sensitive enantiomeric separation method for new single enantiomer drug levornidazole by CD-capillary electrophoresis

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ABSTRACT

A fast and sensitive chiral capillary electrophoresis method has been developed to determine levornidazole and its enantiomeric impurity at a 0.05% level in levornidazole injection solution. Several chemical and instrumental parameters which have an effect on chiral separation were investigated, including chiral selectors, buffer composition and pH, applied voltage, capillary length, temperature and rinsing procedure. After optimizing all the effective parameters, the ideal separation conditions were 20 mM Tris phosphate buffer at pH 2.1, containing 2.0% (w/v) sulfated-α-cyclodextrin with short end injection at 0.5 psi for 5.0 s. Online UV detection was performed at 277 nm. A voltage of 30 kV was applied and the capillary temperature was kept at 25 °C. 2,4,6-triaminopyrimidine was chosen as internal standard to improve the injection precision. The total analysis time is less than 7 min, which is faster than the existing chiral HPLC method (65 min). The validation of the method was performed in terms of factorial analysis, stability of the solution, different cyclodextrin batches study, selectivity, linearity (from 2.5 μg/mL to 6000 μg/mL, $y=0.0015\ x+0.0304$; $R^2=0.9999$ and the residuals were randomly scattered around 0), LOD and LOQ (0.3 and 1.0 μg/mL, respectively), precision and accuracy. The proposed method was then applied to the enantiomeric purity control of the starting material and injection solution of levornidazole (0.5 mg/100 mL).

1. Introduction

Levornidazole ((S)-(-)-1-chloro-3-(-2-methyl-5-nitroimidazole-1-yl) propan-2-ol, see Fig. 1), the S-enantiomer of ornidazole, has been produced as a second generation single-enantiomer drug of ornidazole. Ornidazole is used for the treatment and prophylaxis of infections caused by anaerobic and microaerophilic protozoa and bacteria [1–3]. By reduction of the nitro group to a more reactive amine group, the compound attacks the microbial DNA and leads to degradation of existing DNA [4–6]. The main side effects of ornidazole are nausea, vomiting, spermatotoxicity and central nerve toxicity [3]. Levornidazole has been observed to have similar clinical efficacy as ornidazole, but with fewer associated adverse drug reactions. Several papers related to different pharmacological activities and toxicity of the two enantiomers of ornidazole have appeared [3,7–11]. Therefore, the R-enantiomer of ornidazole should be considered as an enantiomeric impurity in starting material and pharmaceutical formulations of levornidazole.

Chiral separations performed by HPLC remain the workhorse of all enantioseparations, especially on the preparative scale. The use of polysaccharide and cyclodextrin chiral stationary phases (CSPs) continues to dominate the majority of all LC separations using CSPs [12]. Chen et al. [7] established a chiral LC method for the pharmacokinetics study of ornidazole after i.v. administration of the racemic mixture and individual enantiomers in beagle dogs. Ornidazole enantiomers were analyzed on a Chiralcel OB-H column at 310 nm with a mobile phase consisting of hexane-isopropyl alcohol-methyl *t*-butyl ether-glacial acetic acid (90:2:8:0.5 v/v/v). Later, Wang et al. [3] developed a stereoselective LC method to determine the enantiomers of ornidazole in human plasma and urines sample using a Chiralcel OB-H column and hexane-ethanol-glacial acetic acid (94:6:0.08, v/v/v). There is one published paper describing an enantioseparation method with

Abbreviations: CSPs, chiral stationary phases; CDs, cyclodextrins; S-β-CD, sulfated-β-CD; BGE, background electrolyte; HP-β-CD, hydroxypropyl-β-CD; CM-β-CD, Carboxymethyl-β-CD; CE-β-CD, carboxyethyl-β-CD; P-β-CD, phosphate-β-CD; SP-β-CD, sulfopropylated-β-CD; t_m, migration time; API, active pharmaceutical ingredient; R_s, resolution; N, theoretical plate; R^2, coefficient of determination

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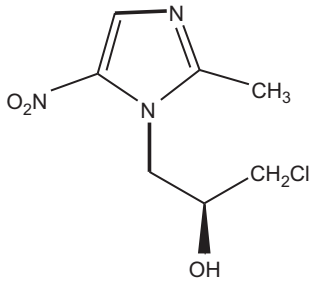

Fig. 1. Structure of levornidazole.

Chiralcel OB-H column and mobile phase hexane-methanol–isopropyl alcohol (95:4:1, v/v/v) to determine the enantiomeric impurity in levornidazole injection and raw material [8]. The elution time of this method is around 65 min. The enantiomeric impurity was not detected in both valid levornidazole injection and raw material. A chromatogram was shown of levornidazole and its chiral impurity at 0.5% level which was not fully baseline resolved. However, these days chiral CE can be considered as an alternative technique to LC in terms of rapid analysis time, lower buffer consumption, high efficiency and use of a wide variety of chiral selectors. Cyclodextrins (CDs) and their derivatives remain the most popular chiral selectors due to their water solubility, stability in solution, UV transparency and ionizability. Chankvetadze et al. [13] investigated the enantioseparation of some chiral pharmaceuticals containing an imidazole (1,3-diazole) moiety, including ornidazole, by using chiral CE. Various native CDs and derivatized CDs were used as chiral buffer additives. As a hydrophobic phenyl moiety is absent in the ornidazole molecule, and, a hydrophilic hydroxyl group is present, none of the used chiral selectors used could resolve the enantiomers of ornidazole. Recently, it has been reported that an enantioselective CE method has been developed for racemic ofloxacin and ornidazole in pharmaceutical formulations using 30 mg/mL of sulfated-β-CD (S-β-CD) and a background electrolyte (BGE) of 50 mM phosphate buffer [14]. The migration time of ornidazole is around 16 min and LOQ is 3 μg/mL.

Since levornidazole has been brought to the pharmaceutical market as a second generation single enantiomer drug, the quality control and enantiomeric impurity control are highly required. Therefore, in our study, a rapid and sensitive enantioseparation CE method was developed for the analysis of levornidazole and its chiral impurity at a 0.05% level. The method was further validated based on ICH guidelines [15]. In addition, the method was applied to bulk sample and injection formulations.

2. Experimental

2.1. Chemicals

Ornidazole reference standard and levornidazole reference standard were kindly donated by Jiangsu Institute for food and drug control (Nanjing, China). Levornidazole bulk sample and levornidazole sodium chloride injection (0.5 g/100 mL) were from Nanjing sanhome pharmaceutical Co., Ltd (Nanjing, China). β-CD and hydroxypropyl-β-CD (HP-β-CD) were purchased from Acros organics (New Jersey, USA). Carboxymethyl-β-CD (CM-β-CD), carboxyethyl-β-CD (CE-β-CD), methyl-β-CD and phosphate-β-CD (P-β-CD) were acquired from Sigma Aldrich (St. Louis, USA). α-CD and γ-CD were obtained from Cyclolab (Budapest, Hungary). Sulfobutylether-β-CD and sulfopropylated-β-CD (SP-β-CD) were obtained from Biotium (Hayward, USA). S-β-CD and S-α-CD were bought from Sigma Aldrich (Bornem, Belgium).

Ortho-phosphoric acid 85% w/w and methanol, HPLC grade were from VWR (Leuven, Belgium). Disodium hydrogen phosphate and sodium hydroxide were bought from Fisher Scientific (Leicestershire, UK) and Tris was purchased from Applichem (Darmstadt, Germany). All reagents were of analytical or LC grade. The water used for preparing solutions was milli-Q water (Millipore, Milford, Massachusetts, USA).

2.2. CE apparatus and conditions

All the experiments were carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter Instruments, CA, USA) equipped with a diode array UV–vis detector. An uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm i.d. and 60.2 cm total length (short end injection, 10.2 cm effective length) was used. Short end injection was performed at the cathodic side which is from the end of the capillary nearest the detector. Instrument control and data evaluation were performed using 32 Karat software (Beckman Coulter, version 5.0). Unless stated otherwise online UV detection was performed at 277 nm, a voltage of 30 kV was applied and the capillary temperature was controlled at 25 °C by liquid cooling. A Metrohm pH meter was used after calibration (Herisau, Switzerland). Sample solutions were introduced by pressure (0.5 psi) for 5 s. Every day, before starting or after finishing the experiments, the capillary should be washed with 0.1 M NaOH for 10 min, followed by H_2O for 10 min. Between each run, the rinsing procedure comprised 0.1 M NaOH for 2 min, then H_2O for 2 min, followed by 3 min BGE.

2.3. Electrolyte and sample preparation

2.3.1. Background electrolyte

For the preparation of the BGE, 2.0% (w/v) S-α-CD was weighed in a flask, dissolved and diluted with 20 mM Tris-phosphate buffer (pH 2.1) to the volume. The 20 mM Tris phosphate buffer was prepared by adding 1 M Tris to a phosphoric acid solution until pH 2.1 was reached. The final phosphate concentration was 20 mM. Refresh the BGE solution after 12 runs.

2.3.2. Sample preparation

Levornidazole reference standard solution: 250.0 mg of the reference substance and a certain amount of 2,4,6-triaminopyrimidine were transferred into a 50.0 ml flask. Dissolve and dilute to volume with H_2O.

Levornidazole sodium chloride injection solution: an appropriate amount of 2,4,6-triaminopyrimidine was dissolved in 50.0 mL of levornidazole sodium chloride solution. The sample solution could be injected after filtration through a 0.2 μm pore size filter.

2.4. Experimental design

A factorial analysis study was performed by means of an experimental design and multivariate analysis using Modde 5.0 software (Umetrics, Umeå, Sweden). A two level full factorial design was used. The number of runs is equal to 2^k+n, where k is the number of parameters and n is the number of center points. Three variables were investigated in this study including concentration of CD, concentration of the BGE and temperature of the capillary. With the number of variables ($k=3$) and 3 replicates of the center point, 11 experiments were performed. The statistical relationship between a response Y and the experimental variables X_i, X_j, … is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \ldots + E$$

where the β's are the regression coefficients and E is the overall experimental error.

The linear coefficients, β_i and β_j, describe the quantitative effect of the experimental variables in the model while the cross coefficient β_{ij} measures the interaction effect between the variables i and j.

3. Results and discussion

3.1. Method development

3.1.1. Screening of selectors

It is well known that the most important step of developing a chiral CE method consists of choosing suitable chiral selectors. In this study, several neutral CDs and negatively charged CDs were screened at different pH values as potential selector candidates, namely α-CD, β-CD, γ-CD, CE-β-CD, HP-β-CD, SBE-β-CD, methyl-β-CD, SP-β-CD, S-β-CD, S-α-CD, CM-β-CD and P-β-CD. After the screening process, no neutral CDs could separate the ornidazole enantiomers (500 μg/mL) at pH 2.0 in a normal polarity mode. On the other hand, four kinds of charged CDs including SBE-β-CD, SP-β-CD, S-β-CD and S-α-CD could give a baseline resolution in a reverse polarity mode. For the enantioseparation with expensive SBE-β-CD and SP-β-CD, large amounts were needed. As previously reported, S-β-CD gave enantiomer separation at lower pH [14] and it was seen in our study that levornidazole eluted before its chiral impurity. However, when applying S-α-CD, the migration order of the two enantiomers was changed, resulting in a good order for an impurity control method with the impurity eluting before the main peak. Moreover, the peak shapes of the two enantiomers improved in comparison with employing S-β-CD and the resolution (R_s) between both peaks was satisfactory. Therefore, 2% of S-α-CD was chosen for further study.

3.1.2. Choosing buffer pH and composition

Based on the nature and structure of ornidazole, the pK_a value is about 2.4 with a weakly basic imidazole group. Therefore, the compound could be treated as a cation at lower pH. The buffer pH was investigated from 1.5–3.1 with a constant 2% of S-α–CD. Below pH 1.9, the baseline fluctuated which would influence the method sensitivity. The problem was due to the lower pH causing a high current. At pH 2.1, there was enough resolution between the two enantiomers and the baseline became stable. When increasing the pH from 2.4 to 3.1, ornidazole could not be detected in reversed polarity mode. When applying normal polarity, ornidazole migrated at 2 min, but without any separation. That is probably because the speed of EOF increased, which carries the analytes–selector complex to the cathode. The high pH also leads to lower interactions between analyte and chiral selectors. The buffer co-ion is also a very important parameter in CE methods since it not only has an influence on current strength, but also represents a good way to avoid peak shape distortions. If the electric field strength in the sample zone and the electric field strength in the pure BGE zone are different, the electrophoretic velocity of the analyte in the sample zone and in the pure BGE zones will be different and lead to fronting or tailing peaks [16]. This phenomenon is known as electromigration dispersion. At a pH around 2, phosphate is the most effective background buffer. Three co-ions, namely, sodium, potassium and Tris, were examined and compared. In contrast to potassium and sodium, Tris is a big molecule which migrates slowly and produces less current. Considering that the pH of BGE is lower and in order to avoid the Joule heating problem during later investigation, Tris-phosphate buffer was used in our study.

3.1.3. Short end injection

For an analytical method, the analysis time should be optimized at the very beginning. This could save time for optimization of other parameters during the method development phase and also plays an important role when the method is used in routine analysis. Usually, the two most convenient ways of getting a rapid method are an increase of applied voltage and a decrease of the capillary length. However, the maximum applied voltage to be used in a commercial instrument is 30 kV and a decrease of capillary length produces higher currents. A short end injection could provide an extremely rapid analysis time without any Joule heating problem. In this study, due to the strong separation power of S-α-CD, R_s of the two enantiomers is large, which provides a chance to perform short end injection. A capillary of 60.2 cm long and 50 μm i.d. was used, and the effective length was 10.2 cm. The ornidazole enantiomers were injected from the end of the capillary nearest the detector giving a total analysis time less than 6 min instead of 15 min for conventional injections.

3.1.4. Buffer concentration

Buffer concentration determines the ionic strength of the BGE and influences the buffer capacity. A range of 10–70 mM of Tris-phosphate buffer was studied. The buffer capacity of the buffer is determined by the actual amounts of acid and salt. A buffer made with very little acid and salt will not have a great buffer capacity while a higher buffer concentration can cause a Joule heating problem. 20 mM Tris-phosphate buffer with short end injection was finally used, because peak shapes, repeatability and electric current remained satisfactory after 20 continuous injections.

3.1.5. Selector concentration

The CDs concentration influences the R_s of the two enantiomers and also has an effect on the migration time. A range from 1.0% to 4.0% of S-α-CD was taken for the study. Theoretically, a higher CD concentration causes an increase of chiral resolution with a maximum. In our case, levornidazole and its chiral impurity in a ratio of 1:1000 (5000 μg/mL levornidazole and 5 μg/mL enantiomeric impurity) were used to investigate if R_s was enough for chiral impurity control. Due to the limited effective capillary length in short end injection, the R_s of the enantiomers slowly increases from 2.0%–4.0%. However, the current was observed to increase obviously. When the concentration of S-α-CD was 2.0%, the R_s was more than 5, which is enough for determining the chiral impurity. The migration time is less than 7 min.

3.1.6. Internal standard

Several compounds which could possibly migrate in front of the two enantiomers, were tried as an IS. Finally, 2,4,6-triaminopyrimidine was selected since the migration time is around 1 min and the peak shape was quite good. The stability of the IS will be investigated.

3.2. Factorial analysis

The purpose of factorial analysis is to investigate the effect of a certain range (indicated at 3 levels by lower, center and higher level) of changes of electrophoretic parameters on target response factors by means of an experimental design. The different electrophoretic parameter settings in the design are concentration of CDs (lower 1.5%, center 2%, higher 2.5%), BGE (lower 15 mM, center 20 mM, higher 25 mM) always at pH 2.1, temperature (lower 20 °C, center 25 °C, higher 30 °C). The investigated responses included R_s between the two enantiomers, migration

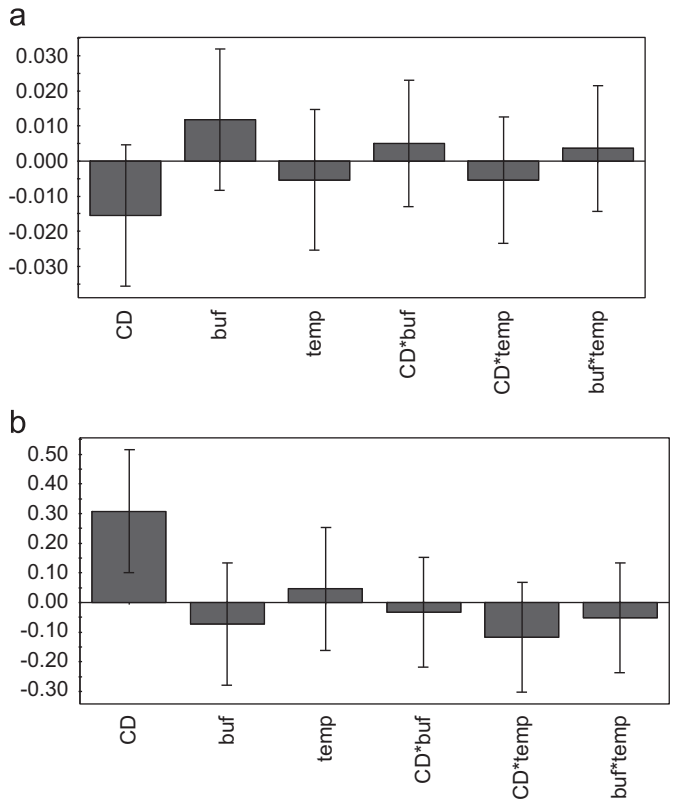

Fig. 2. (a) Regression coefficient plot of impurity peak ratio. (b) Regression coefficient plot of R_s regression Experimental conditions: voltage: 30 kV, pH of BGE: 2.1, short end injection: 5 s at 0.5 psi.

time (t_m) of levornidazole, theoretical plate number (N), current value and the peak ratio (impurity peak/(levornidazole peak+ impurity peak)). Fig. 2a shows the peak ratio response results expressed as a regression coefficient plot. The plot consists of bars, representing the regression coefficients. The bars with one variable reflect the linear effect of that particular variable and the bars with variable 1: variable 2 reflect the interaction between two concerned variables. The line over bars stands for the error (95% confidence interval). All error lines include zero, which means the variation of peak ratio response factor is smaller than the experimental error. Thus the peak ratio change caused by changes of the three electrophoretic variables is considered not to be significant. All the other response factors except R_s were considered to be not significantly influenced by the variables. For R_s (Fig. 2b), it shows that when increasing the CD concentration, R_s increased. However, using 2% (w/v) of CD gave a satisfactory R_s between levornidazole and its chiral impurity. So, improving R_s was not necessary.

3.3. Validation

Validation was performed based on ICH guidelines [15] in terms of linearity, quantitation limit, detection limit, accuracy and precision. The stability of solutions and CD batch effect were also investigated in this section.

3.3.1. LOD and LOQ

The LOD of ornidazole is determined as the concentration yielding a signal three times the noise of the baseline, which in this case equals 0.3 μg/mL (0.006%). The LOQ, which is calculated as ten times the noise, equals 1.0 μg/mL (0.02%). The sensitivity is better than previous one using S-β-CD [14].

3.3.2. Linearity

Since we aimed to develop a method performing assay and purity together as one test, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification. Thus, a calibration curve was constructed with six concentrations corresponding to 2.5, 25, 250, 500, 2500, 6000 μg/mL of levornidazole. Each concentration was injected three times. The regression line was calculated by the method of least squares: $y=0.0015x+0.0304$. Zero was included in the 95% confidence interval of the intercept and the square of the correlation coefficient (R^2) is 0.9999. A residual sum of squares plot was also performed and the residuals were normally scattered around the 0 line.

3.3.3. Accuracy

A recovery test could be assessed on placebo spiked with known amounts of active pharmaceutical ingredient (API) and chiral impurity. Recovery tests of levornidazole and chiral impurity were performed at three different concentration levels. Based on the label of levornidazole injection solution, the placebo solution was prepared by dissolving a certain amount of sodium chloride in water used for preparation of injection formulations, and adjusting the pH to 3.2 with 1 M hydrochloric acid. Since no pure chiral impurity sample was available, ornidazole containing equal amounts of levornidazole and chiral impurity was used to perform the recovery test of enantiomeric impurity. Thus, samples were prepared by dissolving a known quantity of levornidazole reference and ornidazole reference in an amount of placebo. 9 determinations were performed at three concentrations corresponding to 4000, 5000 and 6000 μg/mL of levornidazole and 1 μg/mL, 5 μg/mL and 8 μg/mL of chiral impurity, respectively. With respect to the major analyte, chiral impurity equals 0.02%, 0.1% and 0.16% of levornidazole. Results are shown in Table 1.

3.3.4. Precision

Intraday precision can be deduced from determinations of accuracy. The interday precision was collected over three days (9 determinations at higher, middle and lower concentrations for chiral impurity and levornidazole). The results of interday precision and intraday precision are shown in Table 2.

3.3.5. CD batches effect test

The commercially available S-α-CD consists of numerous isomers, which vary by their substitution degree and also site of substitution. Batch to batch difference may result in different mobility and selectivity, while sometimes, poor reproducibility. In our study, two batches of S-α-CD (batch No. MKBH3350 and MKBD6277) were compared. R_s between the two enantiomers was calculated and compared by a t-test (95% confidence level). It can be concluded that there was no statistically significant difference in resolution yielded by the two batches.

Table 1
Accuracy of levornidazole and enantiomeric impurity. Experimental conditions: see Fig. 3.

Added (μg/mL)	Amount found (μg/mL)	Recovery %
Levornidazole		
4000.0	4140.0	103.5
5000.0	5025.0	100.5
6000.0	6462.0	105.2
Enantiomeric impurity		
1	1.02	102.0
5	5.05	101.0
8	8.22	102.7

Table 2
Intraday and interday precision. Experimental conditions: see Fig. 3.

Concentration (μg/mL)	Intraday precision RSD (%) (from accuracy data)	Interday precision RSD (%) (performed using reference)
Levornidazole		
4000	0.6	2.3
5000	2.4	2.8
6000	1.7	1.1
Chiral impurity		
1	2.6	4.0
5	3.4	3.1
8	3.6	3.1

Table 3
Assay values for several batches of levornidazole raw materials and injections. Experimental conditions: see Fig. 3.

	Assay (%)
Batches of injections	
201011021	100.0
200912041	100.5
201003171	101.3
201201091	100.3
Batches of raw materials	
20091001	99.9
20100501	100.6
20100502	101.4
200906301	101.5

3.3.6. Stability of sample solution

Freshly prepared stock solutions of levornidazole (5000 μg/mL) were compared to 15 days old ones stored at 5 °C in brown bottles. There was no significant difference of relative corrected peak area between fresh and old solutions compared by a *t*-test (95% confidence level). However, to avoid possible interaction with the IS, the IS was prepared freshly and added into the stock solution just before the experiments were performed.

3.3.7. Selectivity

A blank test was performed by checking injection placebo solution. There was no interference. In addition, there was also no interference at different peak sections for levornidazole by using DAD.

3.4. Quantitative results

3.4.1. Assay of levornidazole in injection solution

All calculations on the sample solutions are performed with reference solutions injected prior to the sample solutions. For each batch, two preparations of sample were injected to get three determinations for each. Results are shown in Table 3.

3.4.2. Determination of enantiomeric impurity in injection solution

In order to investigate the capability of developed method, several batches of levornidazole raw materials and injections were tested. No chiral impurity could be detected in injection solution sample (0.5 g/100 mL) or raw materials. When spiking 5 μg/mL racemic ornidazole, which corresponded to 0.05% of levornidazole into injection sample solution, the chiral impurity could be detected and quantified by internal normalization. Fig. 3a shows a typical electropherogram of 5 mg/mL levornidazole and sodium chloride injection solution spiked with 2.5 μg/mL enantiomeric impurity (0.05%). It proves that if chiral impurity is present in the sample solution at a level of 0.05%, the developed method can be applied to quantify the chiral impurity and perform the assay simultaneously.

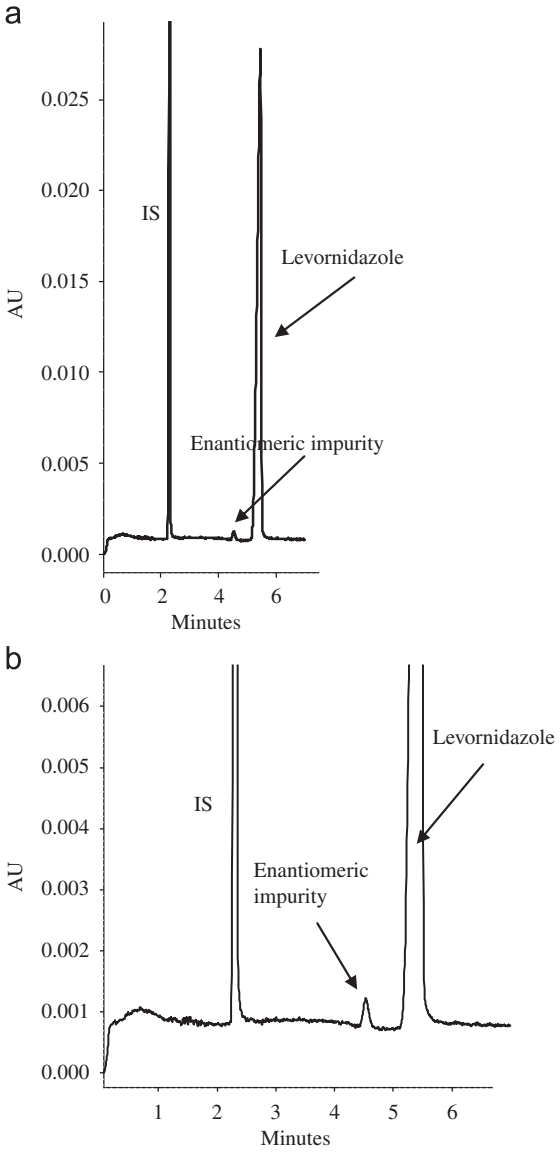

Fig. 3. Typical electropherogram of 5 mg/mL levornidazole injection solution spiked with 2.5 μg/mL enantiomeric impurity (0.05%) (a) full size, (b) enlarged view. Experimental conditions: BGE: 20 mM Tris-phosphate buffer containing 2.0% (w/v) S-α-CD at pH 2.1, voltage: 30 kV, temperature: 25 °C, short end injection: 5 s at 0.5 psi.

4. Concluding remarks

A sensitive and rapid chiral CE method was developed for levornidazole. Applying short end injection, total analysis time is around 7 min, which is much faster than the existing chiral LC method [8]. After validation, the method could be applied to the drug substance and injection solution for the simultaneous determination of levornidazole and its enantiomeric impurity.

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