Xiaolan Deng[1]*
Xin Hai[1]
Ruud Vervoort[2]
Dirk Pamperin[2]
Erwin Adams[1]
Ann Van Schepdael[1]

[1]Laboratorium voor Farmaceutische Analyse, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Leuven, Belgium
[2]Synthon BV, Nijmegen, The Netherlands

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Short Communication

Development and validation of a chiral capillary electrophoresis method for assay and enantiomeric purity control of pramipexole

A rapid method for the enantioseparation of pramipexole and its *R*-enantiomer has been developed by capillary electrophoresis. The influence of chemical and instrumental parameters was investigated including the type and concentration of chiral selectors, buffer composition and pH, co-ions, applied voltage, capillary length and temperature. Optimal separation conditions were obtained using a 50 mM phosphate buffer (pH 2.8) containing 25 mM carboxymethyl-β-cyclodextrin on a fused-silica capillary. Online UV detection was performed at 262 nm. A voltage of 25 kV was applied, and the capillary temperature was kept at 25°C. Hydrodynamic injection was performed at 3.45 kPa for 5.0 s. The separation of enantiomers was achieved in <6.5 min. The method was further validated in terms of stability of solutions, selectivity, linearity (both pramipexole and *R*-enantiomer, $R^2>0.995$), LOD and LOQ (0.91 and 2.94 μg/mL, respectively), repeatability (RSD $<1.5\%$) and accuracy (pramipexole, 100.4%; *R*-enantiomer, 100.5%). The proposed method was then applied to two kinds of pramipexole dihydrochloride monohydrate commercially available tablets, immediate release tablets (1.50 and 0.125 mg) and sustained release tablets (0.52 mg), to quantify the main component in the tablets. The amount of distomer could be quantified in bulk sample materials.

Keywords: Chiral capillary electrophoresis / Enantiomeric purity control / Pramipexole / Quality control
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1 Introduction

Pramipexole (PAL, (6*S*)-6-*N*-propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine, Fig. 1), a synthetic aminobenzothiazole derivative, is a non-ergoline dopamine receptor agonist effective for the treatment of early-stage Parkinson's disease [1–3] and idiopathic restless legs syndrome in adults [4]. PAL selectively acts at dopamine receptors belonging to the D_2 subfamily, where it possesses full activity similar to dopamine itself and exhibits highest affinity for the D_3 receptor subtype probably contributing to efficacy in the treatment of both the motor and psychiatric symptoms of Parkinson's disease, but little or no affinity for the D_1 receptor family [4–7]. In the recent years, PAL has also been investigated as the augmentor of standard antidepressants on treating depression and also decreases the side effects such as sexual dysfunction [8].

PAL has one chiral centre. The *R*-enantiomer presents an approximately 100-fold lower affinity for dopamine receptors in contrast to the *S*-enantiomer, though it has been shown to be efficiently neuroprotective and antioxidative as well as the *S*-enantiomer [3, 6, 7]. Thus, the biological activity is predominantly attributed to the *S*-enantiomer. To improve the efficacy of the drug, PAL is produced as a single pure *S*-form while the *R*-enantiomer, as a chiral impurity, should be detected and limited.

Undoubtedly, capillary electrophoresis (CE) is a very powerful analytical tool and widely applied technique for enantiomeric separation by reason of its chiral resolution power, high separation efficiency and low consumables requirement. Several reviews regarding chiral CE have appeared recently [9–15]. Enantioseparation can be achieved by adding many kinds of chiral selectors such as copper amino acid complexes [16], antibiotics [17, 18], chiral crown

Correspondence: Professor Ann Van Schepdael, Laboratorium voor Farmaceutische Analyse, Katholieke Universiteit Leuven, O&N2, PB 923, Herestraat 49, B-3000 Leuven, Belgium
E-mail: ann.vanschepdael@pharm.kuleuven.be
Fax: +32-16-323448

Abbreviations: CM-β-CD, carboxymethyl-β-CD; **IR**, immediate release; **IS**, internal standard; **PAL**, pramipexole; **RCPA**, relative corrected peak area; **SR**, sustained release; **TEA**, triethanolamine

*Additional correspondence: Xiaolan Deng
E-mail: xiaolandeng20@gmail.com

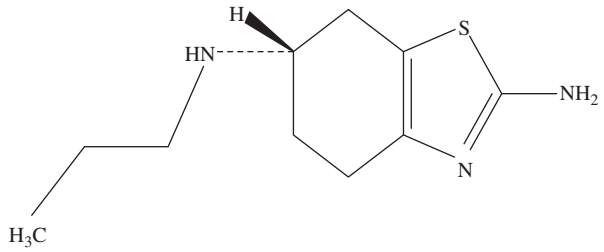

Figure 1. Structure of PAL.

ethers, proteins and polysaccharides [19, 20]. However, cyclodextrins (CDs) and their derivatives are the most frequently used chiral selectors in CE because of their good solubility in aqueous solvents and low ultraviolet (UV) absorbance. The mechanism of chiral separation using CDs is the inclusion of the analytes into the cavity of the chiral selector and interaction with the primary 6-hydroxyl and secondary 2,3-hydroxyl groups, respectively [21, 22]. Moreover, modified CDs exhibit some different properties than the native ones, such as the possibility for different secondary bonds, potential for the analysis of uncharged compounds, and different hydrophobicities of the cavity, which can be easily used for improving the selectivity of the enantiomer separation [22]. Carboxymethyl-β-CD (CM-β-CD) is one of the most frequently used CD derivatives containing chargeable carboxylic groups, and thus their charge depends upon the pH of the running buffer. At low pH (2.5), they are not charged and behave like neutral CDs. At pH above 5, they are completely ionized. These carboxylic functions located on the rim of the CD can enhance the selectivity and chiral resolution, probably due to the hydrogen-bonding capacity of these functions and the higher polarity of these carboxylic functions [23–25].

Several studies have been published for the determination of achiral PAL in biological samples by CE coupled with laser-induced fluorescence detection [26] or liquid chromatography (LC) with electrochemical and UV detection [27] or with atmospheric pressure chemical ionization tandem mass spectrometry [28]. Recently, an experimental design LC analysis of PAL and its impurities was also performed [29]. However, few papers have been presented on the enantioseparation of PAL and its *R*-enantiomer. Only Pathare et al. reported a validated chiral LC method for the enantiomeric purity control of PAL on a Chiralpak AD column and applied it on bulk drugs [30]. To the best of our knowledge, no study has been published describing the chiral separation of PAL with CE till now.

In the present work, a rapid enantiomeric CE method was developed for PAL and its *R*-enantiomer. The influence of chemical and instrumental parameters was first investigated including type and concentration of chiral selectors, buffer composition and pH, co-ions, applied voltage, capillary length and temperature. The optimal separation conditions were further validated for the quantification and enantiomeric purity testing of PAL. The proposed method was then applied to two kinds of active PAL commercially available tablets, immediate release (IR) tablets (1.50 and 0.125 mg) and sustained release (SR) tablets (0.52 mg), to quantify the main component present in the tablets. On the other hand, the distomer was spiked into bulk PAL material containing no *R*-enantiomer to prove that the developed method was able to quantify 0.3% of enantiomeric impurity in bulk material.

2 Materials and methods

2.1 Chemicals

PAL dihydrochloride monohydrate and *R*-enantiomer dihydrochloride monohydrate were kindly donated by Synthon BV. 2,4,6-Triaminopyrimidine as an internal standard (IS) was purchased from Fluka (Steinheim, Germany). 3,5-Diaminobenzoic acid 98% and 1,1-dimethylbiguanide hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All CDs were of CE grade (purity >95%). β-CD and hydroxypropyl-β-CD were purchased from Acros Organics (NJ, USA). Sulfated-β-CD, CM-β-CD, methyl-β-CD and phosphate-β-CD were acquired from Sigma-Aldrich. Carboxyethyl-β-CD, methyl-α-CD, α-CD, CM-α-CD, hydroxypropyl-α-CD and γ-CD were obtained from Cyclolab (Budapest, Hungary). Sulfobutylether-β-CD was obtained from Biotium (Hayward, USA). *Ortho*-phosphoric acid 85% w/w was from VWR (Leuven, Belgium). Acetic acid and triethanolamine (TEA) were acquired from Chem-Lab (Zedelgem, Belgium). Sodium hydroxide (NaOH) was obtained 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.

2.2 CE apparatus and conditions

All the experiments were carried out on a P/ACE MDQ CE 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 75 μm id and 50 cm total length (40 cm effective length) was used. Injection was done at the anodic side and detection was performed at the cathodic side of the capillary. Instrument control and data evaluation were performed using the 32 Karat software (Beckman Coulter, version 5.0). Unless stated otherwise, online UV detection was performed at 262 nm, a voltage of 25 kV was applied and the capillary temperature was controlled at 25°C by liquid cooling. Sample solutions were introduced by pressure (3.45 kPa) for 5 s, and the separation buffer was 50 mM phosphate buffer (pH 2.8) containing 25 mM CM-β-CD.

The relative corrected peak area (RCPA) was used for calculations. RCPA is defined as the ratio of the corrected

peak area of the analyte over the corrected peak area of the IS.

2.3 Sample preparation

2.3.1 Stock solution

PAL dihydrochloride monohydrate was prepared in water at a concentration of 2 mg/mL. Stock solution of *R*-enantiomer dihydrochloride monohydrate (500 μg/mL) was prepared by dissolving the appropriate amount of the substance in water. An IS, 2,4,6-triaminopyrimidine, was prepared at a concentration of 2 mg/mL. All the stock solutions were diluted with water to the concentration needed and stored at 2–8°C in the refrigerator.

2.3.2 Background electrolyte (BGE)

Buffer solution was prepared by mixing a suitable amount of *ortho*-phosphoric acid 85% w/w with water and adjusting the pH value to 2.8 with a 2 M NaOH solution. An appropriate amount of CM-β-CD was dissolved in the buffer to get a concentration of 25 mM. After adding CD, the pH of the solution was found to be 4.5. The BGE is filtered through a 0.2-μm membrane.

2.3.3 Determination of PAL in IR tablets (0.125 and 1.5 mg) and SR tablets (0.52 mg)

The stock solution of PAL dihydrochloride monohydrate was diluted with water to a concentration of 50 μg/mL, and IS was added in a concentration of 20 μg/mL.

For tablet analysis, each kind of tablet was weighed and finely powdered in a mortar separately. Taking into account the average weight, for IR 1.5 mg tablets, an amount of tablet powder corresponding to 250 μg PAL dihydrochloride monohydrate was weighed in a 5.0-mL volumetric flask while for IR 0.125 mg and SR tablets, an amount corresponding to 50 μg was taken due to the viscosity of the excipients. Next, the tablet powder weighed was dissolved in water and 1.0 mL of a 100 μg/mL IS solution was added. The volume was made up to the mark with water. After vortexing (5 min), the sample was centrifuged for 10 min at 14 000 × *g*. The liquid was filtered through a membrane filter of 0.2 μm before injection.

3 Results and discussion

3.1 Method development

3.1.1 Selection of chiral selector

Owing to the pK_a value (9.6) and nature of PAL, it can be treated as a cation below pH 8.0. Seven different neutral CDs: β-CD, methyl-β-CD, hydroxypropyl-β-CD, methyl-α-CD, α-CD, hydroxypropyl-α-CD and γ-CD were screened using a 50 mM phosphate buffer at pH 2.5 with different concentrations of CDs (5–50 mM). At pH 2.5, the influence of the electroosmotic flow (EOF) was low, which could allow a longer time for the interaction of analytes and neutral selectors. However, no chiral separation could be obtained with neutral CDs. Analytes migrated at around 3.5 min with all neutral CDs, which indicated that there was a weak interaction between analytes and neutral CDs. In a next step, seven negatively charged CDs: sulfated-β-CD, β-CD-phosphate, CM-β-CD, carboxyethyl-β-CD, sulfobutylether-β-CD, CM-α-CD and α-CD-phosphate were added one by one to the phosphate buffer at different CD concentrations ranging from 5 to 50 mM. They were screened at different pHs according to the pK_a values of selectors and analytes. Some of the charged CDs have weakly acidic functional groups such as carboxymethyl, carboxyethyl and phosphate, which are charged depending on the pH of the BGE. Three of the charged CDs separated the enantiomers, CM-α-CD, sulfated-β-CD and CM-β-CD. Owing to a strong interaction with sulfated-β-CD, enantiomer peaks could not be observed at normal polarity after 30 min. While at reversed polarity, the two enantiomers were baseline separated at pH 3.0 at a concentration of 25 mM sulfated-β-CD. However, the baseline seriously fluctuated and the current was high (–250 μA). On the other hand, 20 mM CM-α-CD could give an initial chiral separation for the enantiomers, while CM-β-CD caused a complete separation at the same concentration. Finally, considering the separation power and influence of current, CM-β-CD was chosen for further investigation.

3.1.2 Influence of selector concentration (CM-β-CD)

The effect of the selector concentration on the resolution of enantiomers and migration times was studied. A range from 15 to 50 mM of CM-β-CD was taken for the study. With a concentration increasing from 15 to 25 mM, the resolution of enantiomer peaks and migration time rose readily. From 25 to 50 mM, resolution increased slowly but higher current and longer migration time obviously appeared. To avoid a Joule heating problem and long migration time, 25 mM is a better choice. The pH values were checked after different CD concentrations (15–50 mM) were supplemented to the BGE. The pH values were always at 4.5.

3.1.3 Optimization of buffer pH and composition

The behavior of chiral PAL was investigated at pH values ranging from 2.5 to 8 with different buffer compositions. At pH 7–8, 20 mM borate buffer was used, but no separation could be obtained due to fast EOF. The enantiomers could only be separated from pH 2.5 to 4.0 with 20 mM phosphate buffer and acetate buffer solutions. At pH 2.8, the best resolution (Rs = 3.7) could be observed with phosphate buffer. As mentioned above (see Section 2.3.2), the final BGE containing CDs had a pH of 4.5.

3.1.4 Role of buffer co-ions

Three different co-ions were investigated, namely sodium, Tris and TEA. In fact, there was no big influence of the co-ion on resolution and peak shape in our study. However, with Tris and TEA as co-ions, the second migrating enantiomer peak was very near to the EOF, which might cause a problem for quantifying peak areas. Therefore, sodium phosphate was chosen for further consideration.

3.1.5 Buffer concentration

It is well known, the buffer concentration determines the ionic strength of the BGE, which can influence the migration times and resolution of enantiomers. A range of 25–75 mM phosphate buffer was studied. With 25 mM phosphate buffer, clearly peak dispersion occurred. More efficient peaks were observed with a buffer concentration of 50–75 mM. Compared with 75 mM phosphate buffer, 50 mM buffer gave a better resolution and lower current value. Thus, 50 mM was considered due to these advantages.

3.1.6 Other strategies to optimize the method

Other secondary parameters were also investigated in our study. Since a higher sensitivity is needed for the study of purity control, capillary internal diameters of 50 and 75 μm were compared. To get a suitable analysis time and higher efficiency of enantiomer peaks, different capillary lengths were tried (30, 40 and 50 cm), as well as short-end injection. The capillary length of 40 cm × 50 μm showed a good separation and shorter migration time at lower concentration of PAL. However, when increasing the concentration of PAL to 50 μg/mL (for each enantiomer), peak tailing appeared even when adjusting the applied voltage, buffer pH and composition and applying a different temperature. Finally, the capillary with length 40 cm and internal diameter 50 μm was abandoned, and 50 cm × 75 μm was selected for the method with a higher sensitivity and acceptable currents (100 μA).

3.1.7 Final conditions

2,4,6-Triaminopyrimidine, 3,5-diaminobenzoic acid and 1,1-dimethylbiguanide hydrochloride were tested as IS. 2,4,6-Triaminopyrimidine was finally selected as the IS which migrated about 3 min before the two enantiomers. Chiral compounds were identified by spiking a certain amount of PAL in the enantiomer solutions. The first migrating peak was PAL. Actually, this migration order could restrict the quantification of chiral impurities, especially when increasing the concentration of main component. The overloading or peak tailing may minimize the resolution of enantiomers or even cover the impurity peak. As will be mentioned under Section 3.2.4, a maximum of 0.3 mg/mL PAL solution can be injected. Figure 2 shows a typical electropherogram of 0.3 mg/mL PAL dihydrochloride monohydrate spiked with 1% *R*-enantiomer (3 μg/mL) at the final conditions. The analysis time (6.5 min) is shorter than that of the LC method [30].

3.2 Validation

According to the ICH, complete method validation for the assay of the main component and quantification of the enantiomeric impurity were performed.

3.2.1 Stability of solutions

Although the stability of the solutions is not listed separately in the ICH guidelines, it is still necessary to be checked because it indicates the validity of experiments. In our test, the stability of stock solutions of PAL, *R*-enantiomer and IS was studied. Freshly prepared stock solutions of PAL (2 mg/mL) and *R*-enantiomer (500 μg/mL) were compared with the old ones stored at 5°C in brown bottles. The RCPA of stock solutions of PAL and *R*-enantiomer aged 25 days was determined as 15% lower than that of fresh ones. However, there was no significant difference of RCPA between fresh and 10-day-old solutions compared by a *t*-test (95% confidence level). On the other hand, IS stock solution

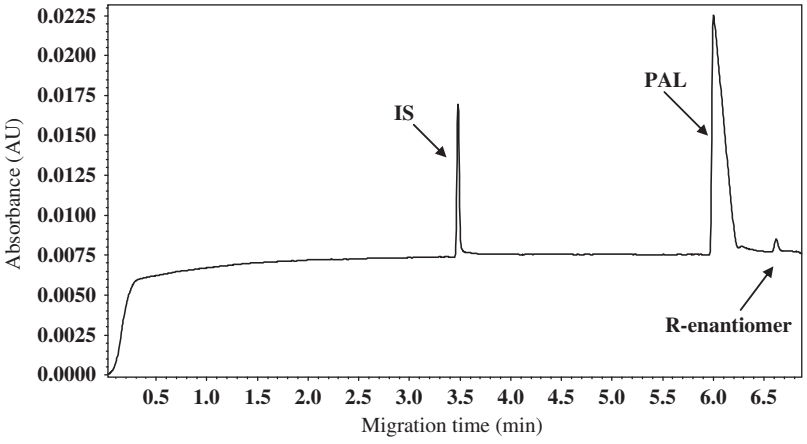

Figure 2. Typical electropherogram of 0.3 mg/mL PAL dihydrochloride monohydrate bulk sample spiked with 3 μg/mL *R*-enantiomer (1%). Experimental conditions: BGE: 50 mM sodium phosphate buffer at pH 2.8 with 25 mM CM-β-CD, voltage: 25 kV, temperature: 25°C, injection: 5 s at 3.45 kPa.

degraded seriously and was only stable for 48 h at room temperature.

3.2.2 Different batches of CDs

The degree and the position of the substitutions on the CD rim can have a great effect on the chiral selectivity of the system. Two batches of CM-β-CD were compared regarding the resolution obtained between two enantiomers. The results were statistically compared using a *t*-test (95% confidence level) assuming equal variances. There was no statistically significant difference between the results obtained with two different batches.

3.2.3 Selectivity

In our study, peak homogeneity was tested first using a diode array detector. There was no interference at different peak sections for PAL, *R*-enantiomer and IS. Selectivity was also tested by injecting tablet placebos (IR and SR). Also here no interference was found.

3.2.4 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of the *R*-enantiomer is determined as three times the noise of the baseline, which in this case equals 0.91 μg/mL. The LOQ, which is calculated as ten times the noise, equals 2.94 μg/mL (RSD = 4.1%, $n = 3$). Owing to peak overloading and limited resolution, a maximum of 0.3 mg/mL of PAL could be injected. Thus, the method is capable of detecting 0.3% chiral impurity. It was attempted to use a concentration of 300 μg/mL of PAL for the tablet preparation, but this solution was too viscous to be injected.

3.2.5 Linearity

For both enantiomers, calibration curves were constructed at five levels over the range from 5 to 75 μg/mL of PAL and 3 to 15 μg/mL of *R*-enantiomer, corresponding to 5, 20, 35, 50 and 75 μg/mL of PAL and 3, 6, 9, 12 and 15 μg/mL of *R*-enantiomer, which is equal to 1, 2, 3, 4 and 5% of a concentration of 0.3 mg/mL PAL dihydrochloride monohydrate as is used for the analysis of bulk samples. Each concentration was injected three times. The regression line was calculated by the method of least squares (for PAL: $y = 0.0129x - 0.0080$, $R^2 = 0.9994$, for *R*-enantiomer: $y = 0.0113x - 0.0010$, $R^2 = 0.9997$, x is the concentration in μg/mL and y is the RCPA). In both cases, zero was included in the 95% confidence interval of the intercept and so it can be concluded that the intercept is not significant. The determination coefficient (R^2) is in both cases higher than 0.995, so good linearity was obtained.

3.2.6 Analytical precision

The intraday precision was performed by using six replicate injections of three standard concentrations for PAL (5, 50 and 75 μg/mL) and *R*-enantiomer (4, 8 and 10 μg/mL) in the same day while interday precision was based on six injections on three consecutive days. The precision obtained for PAL and *R*-enantiomer is summarized in Table 1.

3.2.7 Accuracy

A standard addition method was used for the recovery of assaying PAL dihydrochloride monohydrate tablets. Three different amounts of PAL dihydrochloride monohydrate (40, 50 and 60 μg/mL) spiked into the analytical placebo mainly containing mannitol, corn starch, colloidal silicon dioxide, povidone and magnesium stearate were prepared and analyzed using the procedure described in Section 2.3. Triplicate injections were made for each sample and an average recovery result of 100.4 ± 2.2% was obtained. A linear relationship was found between the added and calculated concentrations ($y = 89.924x + 8.6868$, $R^2 = 0.9997$). The bulk sample of PAL shows no *R*-enantiomer present. Thus, the accuracy of the *R*-enantiomer was carried out by spiking it at lower, middle and higher concentrations in PAL bulk drug samples. Totally, 0.3 mg/mL of PAL dihydrochloride monohydrate bulk drug sample solution was spiked with, respectively, 3, 9, 15 μg/mL of

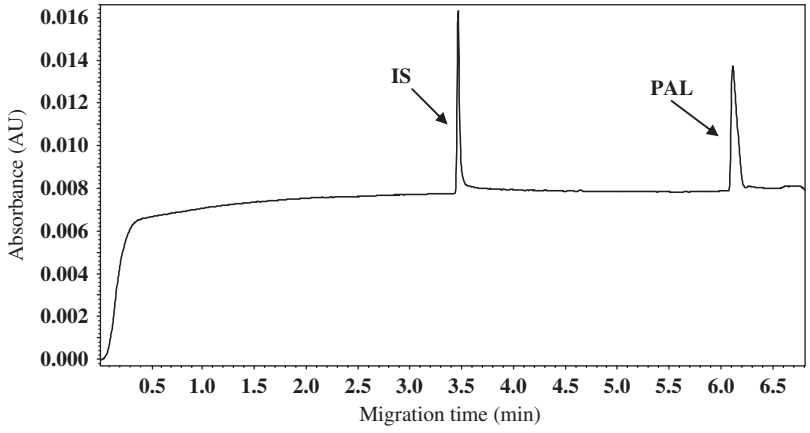

Figure 3. A typical electropherogram of the determination of PAL dihydrochloride monohydrate in tablets. For experimental conditions, see Fig. 2.

Table 1. Analytical precision of PAL and *R*-enantiomer

	PAL			*R*-Enantiomer		
Concentration (μg/mL)	10	50	75	4	8	10
Intraday precision ($n = 6$)						
RCPA[a)] (mean ± SD)	0.1449 ± 0.0008	0.8679 ± 0.0048	1.3361 ± 0.0139	0.0468 ± 0.0007	0.1219 ± 0.0024	0.1681 ± 0.0015
RSD (%)	0.6	0.6	1.1	1.4	2.0	1.2
t_m[b)] (mean ± SD)	4.476 ± 0.0534	4.643 ± 0.0657	4.466 ± 0.0396	4.598 ± 0.0676	4.594 ± 0.0337	4.764 ± 0.0865
RSD (%)	1.1	1.4	0.9	1.3	0.7	1.8
Interday precision ($n = 6$)						
RCPA[a)] (mean ± SD)	0.1507 ± 0.0049	0.8749 ± 0.0154	1.3426 ± 0.0265	0.04814 ± 0.0019	0.1232 ± 0.0058	0.1690 ± 0.0073
RSD (%)	3.2	1.8	2.0	3.9	4.7	4.3
t_m (mean ± SD)	4.485 ± 0.0654	4.656 ± 0.0661	4.389 ± 0.0402	4.568 ± 0.0678	4.592 ± 0.0356	4.773 ± 0.0802
RSD (%)	1.2	1.5	1.0	1.1	0.9	1.8

For experimental conditions: see Fig. 2.
a) RCPA, relative corrected peak area.
b) t_m, migration time.

R-enantiomer dihydrochloride monohydrate. This corresponds to 1, 3 and 5% of impurity present in PAL dihydrochloride monohydrate. Triplicate injections were made for each sample. An average recovery of 100.5 ± 7.3% ($y = 0.0024x + 0.0056$, $R^2 = 0.9988$) was obtained. In both cases, zero was included in the 95% confidence interval of the intercept. These results indicate that the developed method is accurate for this application.

3.3 Quantitative results of tablets

3.3.1 Assay of tablets

The amount of PAL dihydrochloride monohydrate in tablets was quantified. The content of two batches IR (1.5 mg) was 101.0 and 102.3%, and the content of two batches IR (0.125 mg) was 105.4 and 102.2%. The average result of SR (0.52 mg) tablets was 102.5%. A typical electropherogram of the determination of PAL dihydrochloride monohydrate in tablets is shown in Fig. 3.

3.3.2 Comparison with LC method

In comparison with LC [30], this chiral CE method has a shorter analysis time (6.5 min), higher efficiency ($N = 12\,581$) and better tailing factor.

4 Concluding remarks

A fast chiral CE method was developed for the enantioseparation of PAL and its *R*-enantiomer using a simple BGE, 50 mM phosphate buffer (pH 2.8) containing 25 mM CM-β-CD on a fused-silica capillary. Several experimental parameters that influence chiral separation were investigated. The total analysis time is <6.5 min, and the resolution between PAL and *R*-enantiomer is greater than 3. The developed method was further validated for the quantification and enantiomeric purity testing of PAL in terms of stability study of solutions, selectivity, linearity, LOD and LOQ, precision and accuracy. An IS, 2,4,6-triaminopyrimidine, was used to improve the peak area precision. Moreover, with a simple preparation, the main component can be determined by the proposed method in two kinds of PAL dihydrochloride monohydrate commercially available tablets, IR (1.50 and 0.125 mg) and SR (0.52 mg) tablets. Totally, 0.3% *R*-enantiomer in bulk samples of PAL can be detected.

The authors have declared no conflict of interest.

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