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Original Paper

Enantiomeric impurity determination of levetiracetam using capillary electrochromatography

CEC was used to develop a method for the enantiomeric excess determination of levetiracetam, an antiepileptic drug. Different types of calibration curve were evaluated for use in the range between 0.01 and 1 mg/mL when aniracetam was used as an internal standard. The method gave comparable results when only the areas of the impurity were used in the calibration curve. The predicted detection and quantification limits from the S/N were 1.1 and 3.6 μg/mL, respectively. However, experimental results showed that LOD and LOQ were underestimated. Repeatability of injection was demonstrated by the RSD values obtained for retention time, resolution, ratios of the areas impurity/internal standard, and areas of impurity and internal standard individually, which were below or equal to 9.30%. The between-days variability experiments indicated that it is better to make a calibration curve daily. The finally selected calibration curves were used to test the accuracy of the developed method on bulk samples and Keppra® tablets containing 250 mg levetiracetam. Both selected calibration curves performed similarly. The one using the internal standard information gave overall recoveries between 88 and 118%, while the one using areas gave results between 84 and 118%.

Keywords: Capillary electrochromatography / Chiralpak AD-RH / Enantiomeric excess / Levetiracetam

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1 Introduction

Amino acids and sugars occur in nature as single enantiomer forms. Because these molecules are building blocks of enzymes and receptors in the human body, the latter can be seen as a highly chiral environment, in which stereoselectivity will play a crucial role [1]. Administering a racemic drug to a patient is therefore not justified in most of the cases. Therefore, regulatory authorities strongly recommend developing single enantiomer drugs when possible (FDA's policy statement for the development of new stereoisomeric drugs, *US Food and Drug Administration* 1992 (online): http://www.fda.gov/cder/guidance/stereo.htm). In some cases, for example, when it is known that the enantiomer racemizes *in vivo* due to metabolization processes, the development of a racemate can still be approved.

Regarding legislation, three important guidelines are to be followed in the development of a chiral drug (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (online): http://www.ich.org/.). The first is guideline ICH Q6A, which states that if the new drug substance is a single enantiomer form, a chiral identity test, a chiral assay, and a method able to determine the enantiomeric impurity are required for a drug substance. Only the latter two are needed for a drug product. When the drug substance is racemic, it is recommended considering the need for verifying the chiral identity in drug substance release and/or acceptance testing. The second and third guidelines, Q3A and Q3B, concern impurities of regular drug substances. It is stated that enantiomeric impurities are excluded from these guidelines. This originates from the practical difficulties in achieving quantification and identification thresholds, specified in Q3A and Q3B, for chiral impurities similarly as for nonchiral substances. Nevertheless, the principles written in these guidelines are expected to be applied. For drug products

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Abbreviations: CEC, capillary electrochromatography; **WLS,** weighted least squares

containing a nonchiral substance, guideline Q3B specifies that reporting thresholds of the impurities are 0.1 and 0.05%, depending whether less or more than 1 g of the drug is consumed *per* day, respectively.

In this work, capillary electrochromatography (CEC) [2, 3] is used as an analytical separation technique. Because CEC is a relatively new separation technique, few applications are described in the literature dealing with quantitative determinations and method validation [4–8]. Therefore, this technique was used to develop a method able to quantify the enantiomeric impurity in *S*-α-ethyl-2-oxopyrrolidine acetamide, more commonly known as levetiracetam, an antiepileptic drug. The daily dose of levetiracetam is between 1 and 3 g, thus the reporting threshold would be 0.05% for a nonchiral drug impurity. Here, according to Q3B, it is not obliged to reach that level due to the practical difficulties that can occur in the development of chiral separation methods.

Separation and determination of the enantiomeric excess of levetiracetam in formulations have only been realized using LC [9] until now. Here, a Chiralpak AD-H column (amylose tris (3,5-dimethylphenylcarbamate) selector) was used in normal-phase mode to separate the two enantiomers. In the optimized method, the retention times of the *R*-enantiomer and levetiracetam were 9.1 and 13.9 min, respectively, with a resolution above 7, using hexane/isopropanol (90:10) as the mobile phase.

Only one separation of levetiracetam and its *R*-enantiomer has been reported until now using CD-modified microemulsion EKC (MEEKC), an electromigration technique, although a baseline separation could not be achieved [10]. For pure CE, only ionic chiral selectors can be considered to enable the enantioseparation of this neutral compound, as neutral chiral selectors are not able to separate the enantiomers of this substance.

In this paper, it was evaluated whether CEC can give rise to faster analysis and more efficient separations compared to the existing LC method. Based on a generic separation strategy for chiral compounds [11–13], the separation of both enantiomers was optimized and used for the development of the assay for *R*-α-ethyl-2-oxopyrrolidine acetamide. The optimized method was validated, and then applied on the pharmaceutical formulation Keppra® to check its usefulness.

2 Experimental

2.1 CEC

CEC experiments were performed on a Beckman P/ACE MDQ instrument (Fullerton, CA, USA) with a diode array-UV detector, set at 214 nm. The Beckman 32 Karat software version 4.01 (1999-2000 Beckman Coulter) allowed instrument control. The temperature was set at 20°C and controlled by means of liquid cooling.

Electrokinetic injections of the samples were done at 5 kV during 10 s; a mobile phase plug was injected at 5 kV for 5 s to prevent back-migration of the sample. All analyses were performed at 10 kV with a pressure of 5.5 bar (80 psi) on both vials.

To load the mobile phase, the column was rinsed at least 45 min using a flow-splitted L-6000 HPLC pump (Merck-Hitachi, Tokyo, Japan) at approximately 100 bar pressure.

2.2 Columns

Columns with 20 cm packed length and 31.2 cm total length were made using 100 μm ID fused-silica capillary from Composite Metal Services (Ilkley, UK). Two stationary phases, Chiralcel OD-RH (kind gift from Chiral Technologies, Illkirch, France) and Chiralpak AD-RH (Daicel, Tokyo, Japan), were packed into the capillaries, which were sealed with a temporary frit at one side, using a slurry-based method. Briefly, a slurry of 30 mg/mL stationary phase in ACN was forced into the capillary using a pressure of 600 bar, generated by an air-driven liquid pump (Haskel, Burbank, CA, USA). After packing, the column was rinsed with ACN for 3.5 h. Then, two frits were fabricated at 20 cm from each other, by local heating of the stationary phase using a capillary burner (Capital HPLC, Broxburn, West Lothian, Scotland), while flushing with Milli-Q water under a pressure of 350 bars. The same device was used for the fabrication of the detection window.

2.3 Mobile phase and sample solutions

The electrolyte consisted of 5 mM disodium hydrogen phosphate (Na_2HPO_4) from Merck (Darmstadt, Germany), titrated to pH 11.5 with 1 M NaOH (Merck). The mobile phase consisted of this electrolyte solution mixed with ACN (HPLC grade, Fisher, Leicestershire, UK), in a 30:70 v/v ratio. Before use, the mobile phase was filtered over a 0.2 μm FP-Vericel Membrane filter (Pall, East Hills, NY, USA) and degassed during 30 min.

S(−)Levetiracetam and *R*(+)-α-ethyl-2-oxopyrrolidine acetamide were kind gifts from UCB (Brussels, Belgium). Aniracetam was purchased at Sigma (Steinheim, Germany). For the calibration curve, stock solutions of 4 mg/mL of *R*-α-ethyl-2-oxopyrrolidine acetamide and 4 mg/mL levetiracetam together with 0.58 mg/mL aniracetam in mobile phase were used. A constant volume (100 μL) of the solution with levetiracetam and aniracetam was mixed with a variable volume of the stock of the impurity to obtain the appropriate concentration, and further diluted to a volume of 200 μL with the mobile phase. The

final concentrations of levetiracetam and aniracetam in the standards were 2 and 0.29 mg/mL, respectively.

To analyze the tablets, a stock solution of 1.16 mg/mL aniracetam in ACN and the same *R*-α-ethyl-2-oxopyrrolidine stock solution as above were used. Keppra tablets are commercialized by UCB and each tablet contains 250 mg of levetiracetam. To extract levetiracetam from the tablet matrix, two tablets were dissolved in 20.0 mL of mobile phase. This solution was ultrasonicated for 30 min, centrifuged during 25 min at 3000 rpm, and the supernatant was first filtered over an S&S Faltenfilter (Dassel, Germany), followed by a second filtration through a 0.2 μm syringe filter (VWR International, USA). To determine the accuracy of the method, 100 μL of the tablet solution was mixed with the required volume of *R*-enantiomer stock solution, 50 μL of internal standard solution, and further diluted to 200 μL with mobile phase. The final concentrations of levetiracetam and internal standard were 12.5 and 0.29 mg/mL, respectively. The final concentrations of *R*-enantiomer added were 0.01, 0.05, and 1 mg/mL.

3 Results and discussion

In each Keppra tablet, 250 mg of levetiracetam is present. According to the extraction protocol, the final concentration of levetiracetam in the sample solution is 12.5 mg/mL. Following the guideline Q3B, a reporting threshold of 0.05%, an identification threshold of 0.1%, and a qualification threshold of 0.15% should be achieved. The latter is not of importance here, as a qualification threshold implies the acquisition and evaluation of data using this concentration on test subjects to establish biological safety. A reporting threshold of 0.05% corresponds to a concentration of 0.006 mg/mL of the *R*-enantiomer in the sample solution, and an identification threshold of 0.1% to 0.0125 mg/mL.

3.1 Optimization of the separation conditions

Levetiracetam, displayed in Fig. 1, has a calculated pK_a value of 15.74 on the terminal amide group. Therefore, it cannot be ionized in the pH range between 0 and 14, and can be classified amongst the neutral drug molecules. Its log *P* value is −0.67, which means that it is a polar, hydrophilic molecule. Both pK_a and log *P* values were calculated with the ACD Labs version 6.0 (Advanced Chemistry Development, 1994–2002).

According to a previously defined strategy for the chiral separation of non-acidic compounds [12], levetiracetam should be analyzed in a screening step on Chiralpak AD-RH and Chiralcel OD-RH stationary phases using a mobile phase consisting of 70% v/v ACN and 30% v/v of a 5 mM phosphate buffer pH 11.5, at a voltage of 15 kV and at

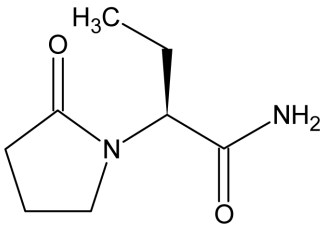


Figure 1. Structure of levetiracetam.

25°C. All experiments were executed with a concentration of 0.25 mg/mL of both enantiomers.

On Chiralcel OD-RH, no separation was seen, but on Chiralpak AD-RH, a nearly baseline separation (resolution of 1.51) was obtained at these conditions. In the next step of the strategy [12], it is recommended to execute a 2^2 full factorial design, *i.e.*, performing four experiments, in which the ACN content of the mobile phase is reduced either to 65 and 55% and the applied voltage to 10 and 5 kV on the column where a partial separation occurred, here Chiralpak AD-RH. However, the separation worsened in all the four experiments, which was generally not expected. Using the initial mobile phase and reducing the applied voltage to 10 kV and the temperature to 20°C had a positive result on the separation. Between the enantiomers, a resolution above 1.7 was achieved, and therefore these conditions were used in further experiments.

Electrokinetic injection was applied to introduce the sample onto the column. Pressurized injections were not possible, as the upper pressure limit is still too low to inject the sample through the inlet frit. Regarding injection time, 30 s was initially used because it is often difficult to inject neutral compounds electrokinetically. Later, it was seen that 30 s caused negative effects such as a decrease of the resolution and nonrepeatable results. Therefore, the injection time was lowered to 10 s in the further experiments.

3.2 Elution order, detection limit (LOD), and quantification limit (LOQ)

The elution order was determined using different concentrations of each enantiomer. It was seen that the impurity eluted before levetiracetam at the selected conditions. This is considered advantageous for impurity determinations because, in the opposite situation, the main compound's peak can hide the impurity or the latter elutes at the tail of the main peak. A typical electrochromatogram is displayed in Fig. 2. All compounds are eluted within 7 min, which can be considered as an advantage. Thus, the analyses are finished quite fast compared with the LC method [9] where the analysis time was 13.9 min.

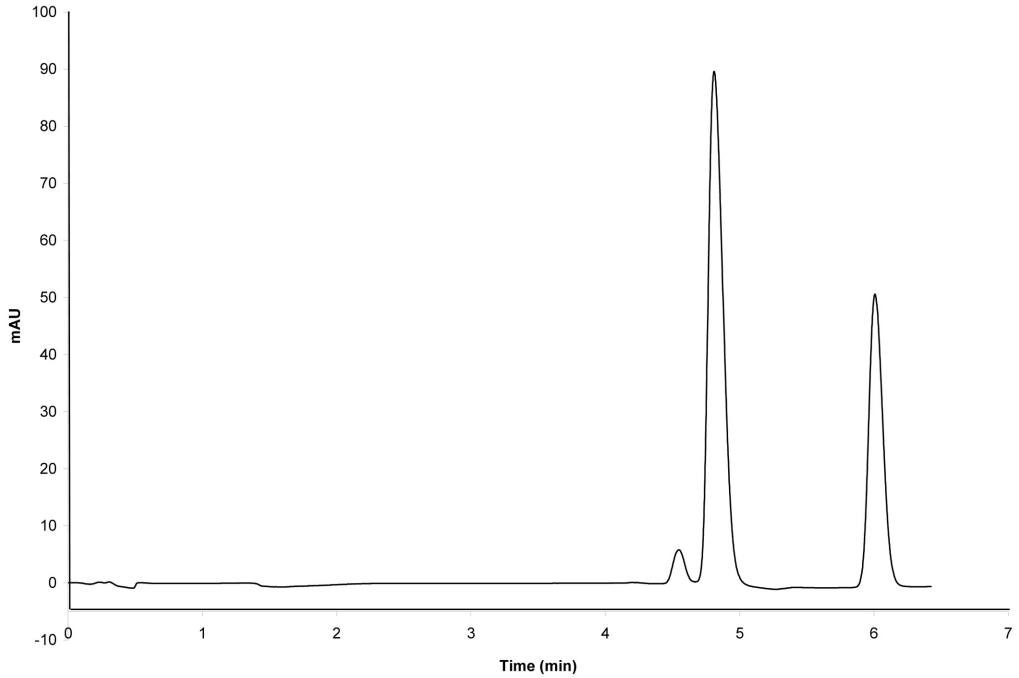


Figure 2. Typical electrochromatogram, obtained at optimal analyzing conditions. From left to right: *R*-α-ethyl-2-oxopyrrolidine, levetiracetam, and aniracetam. Sample: 0.1 mg/mL (0.8%) *R*-enantiomer, 2 mg/mL levetiracetam, and 0.29 mg/mL aniracetam in mobile phase. Experimental conditions: column: Chiralpak AD-RH, mobile phase: 5 mM phosphate buffer pH 11.5/ACN (30:70), applied voltage: 10 kV, temperature 20°C, injection: 5 kV 10 s, and detection at 214 nm.

The detection- and quantification limits were determined by injecting consecutively solutions containing 0.5, 0.05, 0.025, 0.01, and 0.0025 mg/mL of both the enantiomers. At the lowest concentration, two little peaks could be observed (S/N = 4.3), but no straightforward interpretation was possible because the peaks could not be distinguished properly from the baseline noise. The S/N determined using a 0.01 mg/mL concentration of the impurity was 28 and this resulted in a predicted LOD of 0.0011 mg/mL and an LOQ of 0.0036 mg/mL with S/N values of 3 and 10, respectively. However, it was seen that a concentration of 0.0025 mg/mL of both the enantiomers did not give an interpretable result. Therefore, it can be concluded that the calculated LOD and LOQ are underestimated. Hence, the lower limit in the calibration curve was taken as 0.01 mg/mL of *R*-α-ethyl-2-oxopyrrolidine, corresponding to 0.08% of the impurity, relative to the final concentration of levetiracetam in the sample solution. The lower limit of the calibration curve was taken a lot higher than the predicted LOQ because of the underestimation observed. The area precision for three injections is 7.68% for this concentration, which is acceptably good. Therefore, it can be assumed that this is above but approaching the real LOQ, and hence can be accepted as the lowest concentration in our calibration curve. The accuracy obtained at this concentration is reported further. It can already be noticed that the reporting threshold of 0.05% will not be achieved. However, 0.08% is a relatively good result, given the fact that electrokinetic injection is performed to introduce the neutral compound into the column and given the loss of some sensitivity due to on-column detection.

3.3 Calibration curve

Because the use of electrokinetic injections can give rise to variability in results, it was chosen to work with an internal standard for quantitative determinations. Different nonchiral substances such as diazepam, piracetam, and aniracetam were tested. The latter gave the best results as it was well separated from both the other peaks (Fig. 2).

In the calibration curve, levetiracetam was always analyzed together with the impurity to check if the resolution between both the enantiomers remained sufficient. Levetiracetam was analyzed at a concentration of only 2 mg/mL due to a limited availability of the substance. At higher concentrations (above 0.08 mg/mL ~ 0.64%) of the impurity, the baseline resolution was sometimes lost, but it never went below 1.40 (see Fig. 3).

The concentration of the internal standard was set constant on 0.29 mg/mL because its signal was approximately half of that of levetiracetam. For impurity deter-

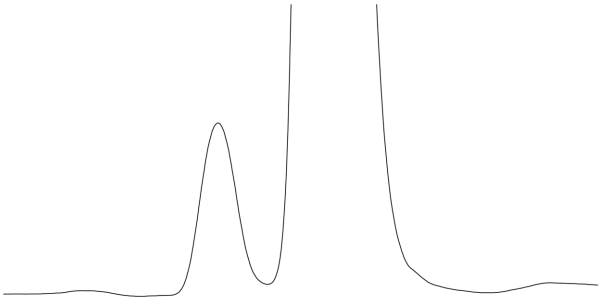


Figure 3. Zoom of the electrochromatogram of the separation between both enantiomers when a resolution value of 1.40 was obtained. Concentration *R*-enantiomer 0.8% with respect to levetiracetam.

minations a lower concentration might have been more appropriate.

The linearity of the calibration curve was checked between 0.01 mg/mL (~0.08%) and 1 mg/mL (~8%) *R*-enantiomer. All standards of the calibration curve were injected three times and the average area was used in further calculations. Using the ratio of the areas of *R*-α-ethyl-2-oxopyrrolidine and aniracetam as a function of the effective concentration, a calibration curve (CCa) was obtained with a correlation coefficient of 0.9995. The equation of CCa was $y = 0.9411x + 0.0012$. When the average impurity area was used, a correlation coefficient of 0.9991 was obtained (CCb), and the data are described by the relation $y = 313\,017x + 2315$. Both calibration lines were subjected to a lack-of-fit test at $\alpha = 0.05$ level and the results indicated are presented in Table 1. For CCb, the calculated *F*-value was smaller than the critical *F*-value, meaning that the linear relationship is adequate to describe the relationship between *x* and *y*. However, for CCa, the *F* value was slightly larger than *F* critical, thus the proposed linear relationship is less good to describe the relation between *x* and *y*. A quadratic model, having an equation of $y = -0.0474x^2 + 0.9879x - 0.0024$, was evaluated as a potentially better model. The significance of the quadratic term was tested and found to be different from zero, meaning that this model was more appropriate. However, a lack-of-fit test again was significant indicating that this calibration curve was still not so suitable. Moreover, the predicted concentrations for spiked samples were not better than those obtained from the line CCa (see Table 2).

A check for linearity was also performed among the lowest concentrations only, *i.e.*, between 0.01 mg/mL (~0.08%) and 0.1 mg/mL (~0.8%). Good correlation was

Table 1. ANOVA analysis of calibration lines CCa and CCb

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-Value	*F*–Critical
CCa ($y = 0.9411x + 0.0012$)					
Due to regression	2.251	1	2.251		
Residual	2.107E−03	22			
Lack-of-fit	1.301E−03	6	2.169E-04	4.310	2.74
Pure error	8.053E−04	16	5.033E-05		
Total	2.253	23			
CCb ($y = 313\,017x + 2315$)					
Due to regression	2.490E + 11	1	2.490E+11		
Residual	1.820E + 07	22			
Lack-of-fit	2.323E + 07	6	3.871E+07	0.390	2.74
Pure error	1.588E + 07	16	9.925E+07		
Total	2.508E + 11	23			

Table 2. Percent recoveries of three spiked tablet samples from the various calibration curves. Origin of Eq. (1–6): see text

Equation calibration line		Concentration *R* 0.08% (0.01 mg/mL)			0.4% (0.05 mg/mL)			8% (1 mg/mL)		
1	$y = 0.9411x + 0.0012$ (Cca)	82.03	81.36	74.26	85.65	104.14	98.53	112.09	90.02	85.70	87.76
2	$y = 313\,017x - 2315$ (CCb)	177.14	174.58	167.47	174.93	127.53	120.03	134.20	99.22	92.05	89.84
3	$y = -0.0474x2 + 0.9879x - 0.0024$	118.19	107.33	114.10	114.74	114.45	101.46	106.83	87.65	85.50	89.99
4	$y = 0.9585x - 0.0028$	125.93	114.75	121.72	122.37	118.48	105.16	110.66	NA	NA	NA
5	$y = 0.9440x - 0.0019$	118.04	106.68	113.76	114.42	118.33	104.81	110.40	89.62	87.52	91.91
6	$y = 334\,257.6x - 718.579$	115.94	108.95	115.61	118.01	116.16	102.88	109.91	83.66	85.72	92.44

NA: Not applicable.

observed when the ratios were used (R^2 = 0.9993), with an equation of $y = 0.9585x - 0.0028$. This calibration line was not significant in a lack-of-fit test. However, the bias of the predicted concentrations from this calibration line was again similar or larger than these obtained for CCa. Therefore, finally CCa remains preferred from a practical point of view.

The above strange observations with both the straight and curved lines (significant lack-of-fit test) can be explained by the fact that the variance of the replicated measurements was low compared to the residuals from the calibration curve, which easily results in statistical significant conclusions but which was of limited practical relevance.

When linearity check was performed among the lowest concentrations for CCb, larger residuals from the straight line could be seen, which is an indication of the advantage of using an internal standard.

The homo- or heteroscedasticity of the data was evaluated using a Cochran's test [14]. For CCa, heteroscedasticity was confirmed and a calibration curve using weighted least squares (WLS) [14] was made with an equation of $y = 0.9440x - 0.0019$. Subjecting this curve to a lack-of-fit test resulted in no significant *F*-value. The predicted recoveries from this curve were also more accurate for lower concentrations than those from CCa (Table 2); for higher concentrations similar results were obtained. Therefore, in the rather broad calibration range of 0.01 – 1 mg/mL, data are best modeled by WLS regression.

For CCb, heteroscedasticity was also confirmed, and a calibration line with equation of $y = 334\,257.6x - 718.579$ was obtained through the application of WLS. A lack-of-fit test was negative. The predictions from this calibration line for spiked tablet samples are given in Table 2. This latter line gives much better predictions than CCb, and is preferred as calibration curve.

3.4 Repeatability

The repeatability was evaluated by analyzing ten times a solution containing 0.1 mg/mL (~0.8%) *R*-α-ethyl-2-oxopyrrolidine, 2 mg/mL levetiracetam, and 0.29 mg/mL aniracetam. The results are displayed in Table 3. The use of an internal standard reduces the injection variability. The RSD is 9.30% for the area of the *R*-enantiomer, whereas it is reduced to 4.28% when the area ratios are used. For the retention time, very good repeatability was observed. For resolution, a somewhat high RSD value (2.94%) was observed, and it could also be seen in the experiments that resolution decreased somewhat as a function of time. A possible explanation for this observation can be given by the fact that the silica of the CSP probably dissolves gradually working under these extreme conditions, with the loss of some chiral selector and enantioresolution as a consequence. However, it was already shown in Section 3.3 that a resolution of 1.40 does not have to be catastrophic for peak integration. However, for quantification purposes, the evolution of resolution over time is to be followed and when the integration of the impurity peak becomes affected the column should be replaced.

Table 3. RSD values calculated for injection repeatability

	%RSD (n = 10)
Area *R*	9.30
Area IS	5.91
Ratio area *R*/area IS	4.28
t_R, *R*	0.68
t_R, levetiracetam	0.73
t_R, IS	0.58
R_s	2.94

IS: Internal standard, *R* = *R*-α-ethyl-2-oxopyrrolidine acetamide.
Experimental conditions: Chiralpak AD-RH, mobile phase: 5 mM phosphate buffer pH 11.5/ACN (30:70), applied voltage: 10 kV, temperature 20°C, injection: 5 kV 10 s, detection at 214 nm. A standard solution containing 0.1 mg/mL (0.8%) *R*-enantiomer, 2 mg/mL levetiracetam, and 0.29 mg/mL aniracetam in mobile phase was used.

3.5 Intermediate precision

The between-days variability and repeatability were evaluated by the duplicated injection of three concentrations during six consecutive days. The RSD values are given in Table 4. For retention time, all between-day RSD were below or equal to 1.92%. For the areas, a larger deviation is observed with RSD values between 7.87 and 14.36%. These results seem quite acceptable in a between-day context. For resolution and the ratio of the areas, the variability is also relatively small. Table 4 also displays the RSD for repeatability estimated as the pooled variance from the duplicated daily injection variances. When we compare with Table 3, for most responses, the values are relatively similar. Comparing the repeatability and the between-days variability, the RSD values of the latter are higher, which was expected. For the ratio area *R*/area IS however, the RSD of the between-days variability is approximately double the repeatability. This indicates that it is better to make a daily calibration curve.

3.6 Accuracy

The results used for the repeatability evaluation were used to determine the accuracy of the calibration curves for bulk samples. Using calibration curve five (Table 2), recoveries between 96 and 110% were obtained for a spiked concentration of 0.8%. Using calibration curve six, recoveries between 84 and 112% were obtained, indi-

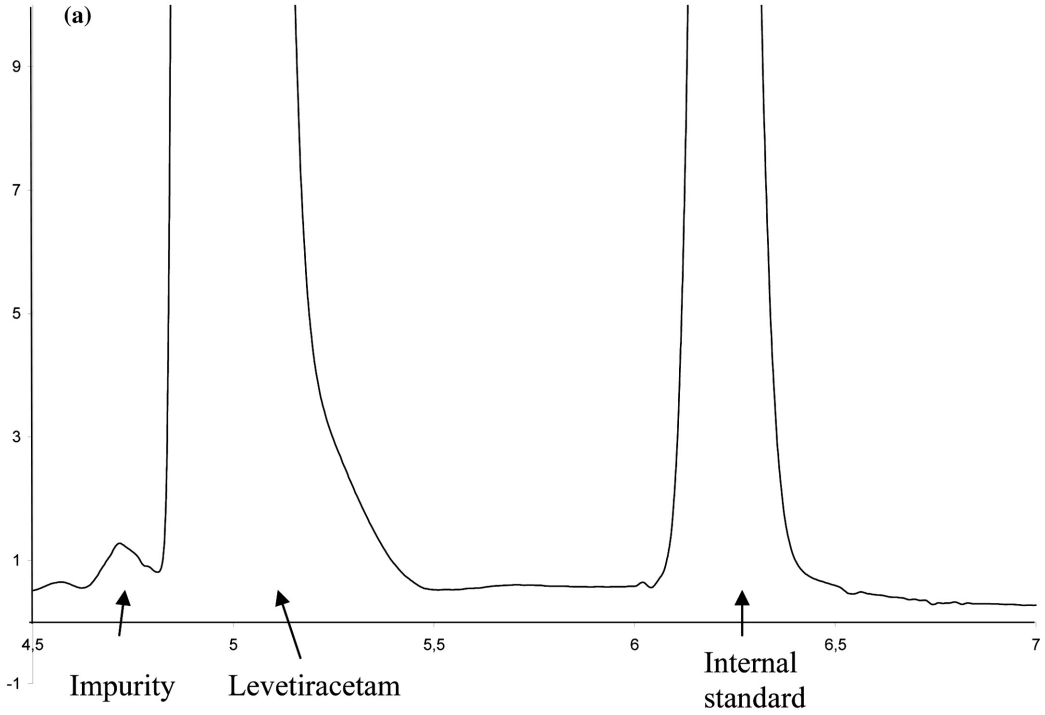
Table 4. Between-days variability (6 days, $n = 6$) and repeatability, expressed as % RSD, for three concentrations

Response	Substance	Concentration *R*		
		0.01 mg/mL = 0.08%	0.1 mg/mL = 0.8%	1 mg/mL = 8%
Repeatability				
Retention time	*R*	0.35	0.28	0.33
	Leve	0.37	0.31	0.33
	IS	0.35	0.23	0.33
Areas	*R*	8.57	10.62	5.85
	Leve	8.84	11.25	7.06
	IS	9.06	9.15	2.46
Resolution		5.20	4.90	4.46
Area *R*/area IS		9.79	3.86	4.61
Between-days variability				
Retention time	*R*	1.26	1.11	1.26
	Leve	1.19	1.04	1.19
	IS	1.92	1.65	1.50
Areas	*R*	10.75	14.36	8.37
	Leve	10.29	11.12	7.87
	IS	9.00	11.80	9.70
Resolution		6.32	5.79	3.01
Area R/area IS		7.91	8.45	5.81

IS: Internal standard, R = *R*-α-ethyl-2-oxopyrrolidine acetamide.
Experimental conditions: Chiralpak AD-RH, mobile phase: 5 mM phosphate buffer pH 11.5/ACN (30:70), applied voltage: 10 kV, temperature 20°C, injection: 5 kV 10 s, detection at 214 nm. A standard solution containing the indicated concentration *R*-enantiomer, 2 mg/mL levetiracetam and 0.29 mg/mL aniracetam in mobile phase was used.

cating a larger variability in results. This is another criterion to prefer the calibration curve based on the ratios.

For the determination of the impurity in tablet formulations, concentrations representing 8, 0.4, and 0.08% of impurity were spiked, of which the obtained electrochromatograms are given in Fig. 4. The recoveries using the different calibration models are displayed in Table 2. For the calibration curves using the ratios (1, 3, 4, and 5), the biases were rather similar for all calibration models,



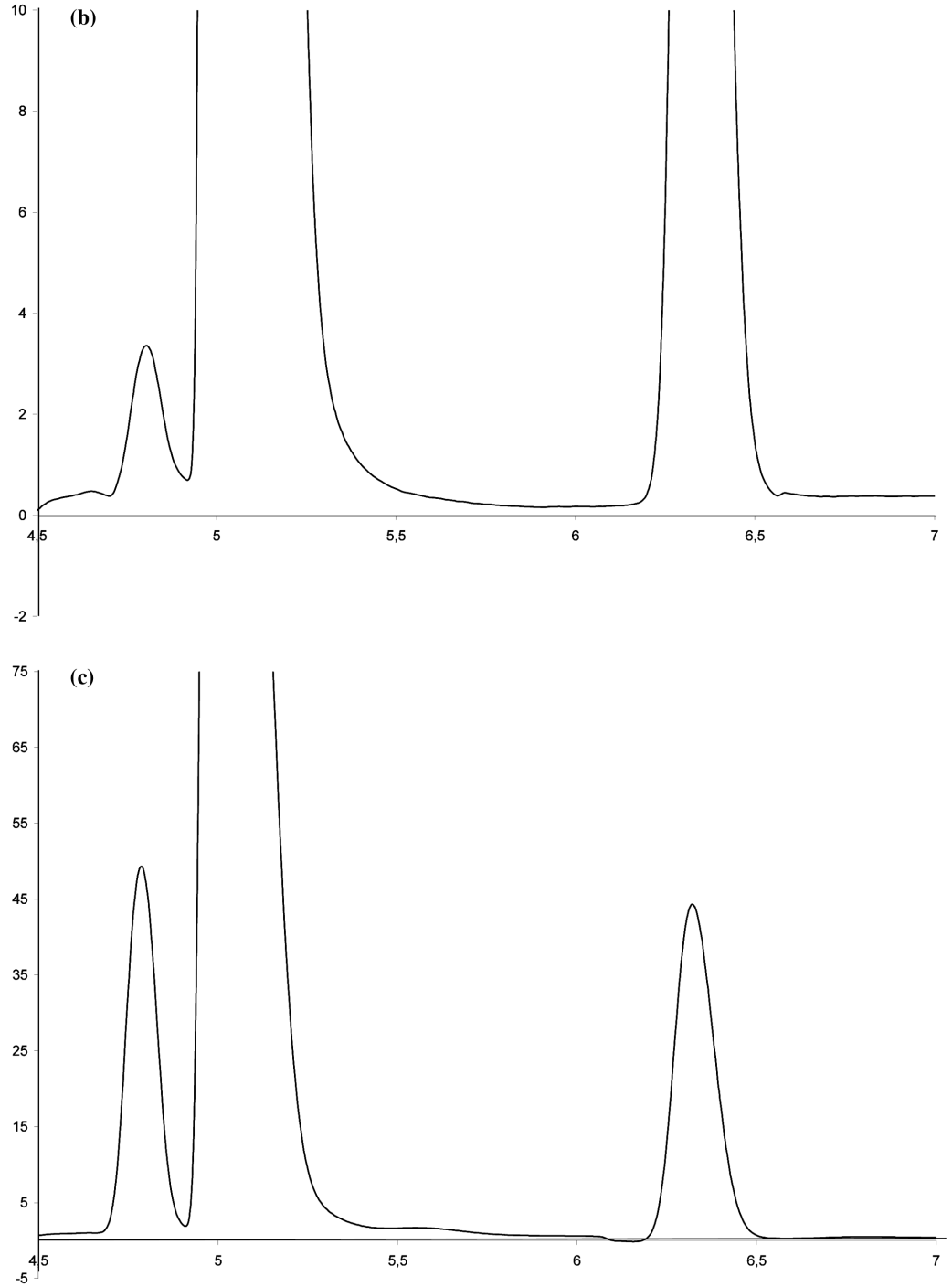


Figure 4. Zoom-ins of the electropherograms of the analysis of *R*-enantiomer-spiked tablet samples. Concentration *R*-α-ethyl-2-oxopyrrolidine (a) 0.08%, (b) 0.4%, and (c) 8%.

except for 5 where it is somewhat smaller for lower concentrations. A remarkable fact is that CCa gives an underestimation at the lowest concentration, while the other lines overestimate. Calibration line number 5 gives the overall best predictions and is considered the best choice. Using this WLS calibration line, recoveries between 88 and 92% were obtained for an 8% spiked sample, and between 105 and 118% were seen for a 0.4% spiked tablet. For the lowest spiked concentration, 0.08%, recoveries between 107 and 118% were found.

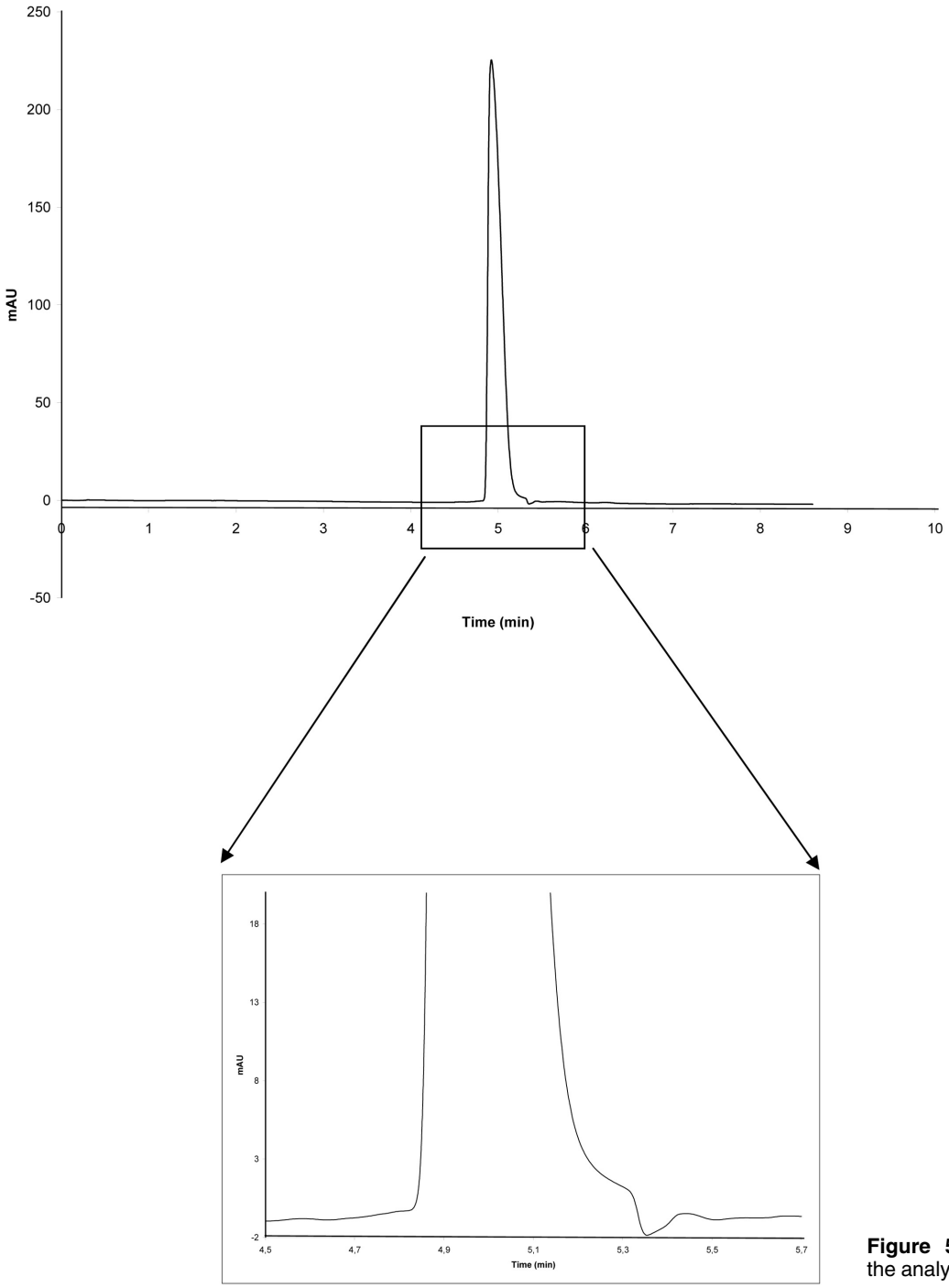


Figure 5. Electropherogram of the analysis of Keppra tablets.

Using the impurity area, the WLS calibration line resulted in predicted concentrations between 109 and 118% for 0.08% impurity, between 103 and 116% for 0.4% impurity, and finally between 84 and 92% for 8% *R*-enantiomer.

The biases found for the ratio-based calibration line are in the same range as for the one using area, thus no preference is given.

These results seem moderate analytically seen, but are quite acceptable given the fact that quantitative determinations using CEC are not frequently reported due to experimental problems and variability.

Summarized, it can be said that the internal standard-based calibration curve results in relatively accurate predictions starting from 0.08% of the *R*-enantiomer, *R*-α-ethyl-2-oxopyrrolidine acetamide, with respect to the

therapeutically active *S*-enantiomer. The use of only the peak area of the *R*-enantiomer in the quantification gave similar results for this case study.

3.7 Application to Keppra tablets

As already mentioned in Section 2, two tablets each containing 250 mg levetiracetam were crushed and dissolved in 20.0 mL of mobile phase, resulting in a concentration of 25 mg/mL of active compound. This solution was diluted twice, obtaining a final concentration of 12.5 mg/mL levetiracetam. Different dissolution procedures have been tested. First, a literature-based procedure [9] was tested, using isopropanol to dissolve levetiracetam from its matrix. However, this solvent caused broad peaks of the internal standard during analysis, and was therefore replaced by water, as levetiracetam is readily soluble in this solvent. Using water as solvent, the internal standard precipitated, and the injections were not reproducible anymore. When the mobile phase was used as the solvent, the aniracetam areas were comparable to those measuring the calibration line. The area of the levetiracetam peak obtained after treatment with the mobile phase was compared with that obtained after treatment with water, in which it can be completely dissolved. Of course, it would be better to compare also with a solution containing 12.5 mg/mL of levetiracetam standard, but this was not possible due to a limited standard availability. It was seen that the peaks of both the extracts were practically identical, *i.e.*, similar height and area, indicating that all levetiracetam from the tablets was present in the resulting solution. Regarding the possible impurity in the tablet, we assume that it is dissolved together with levetiracetam, as both are enantiomers.

No presence of the *R*-enantiomer above the LOD was observed in the Keppra tablets when the obtained solution was injected (Fig. 5). However, the lowest concentration of *R*-enantiomer in the calibration curve (*i.e.*, 0.08%) spiked to a tablet was still separated and quantifiable (see accuracy experiments) in the presence of 100% (~12.5 mg/mL) levetiracetam (Fig. 5a), which indicated the practical utility of the developed CEC method for the enantiomeric excess determination in tablet formulations.

4 Concluding remarks

Using CEC as an analytical separation technique, a method was developed for the quantification of *R*-α-ethyl-2-oxopyrrolidine in levetiracetam tablets. The optimized analyzing conditions consisted of an injection of 5 kV during 10 s, a 5 mM phosphate buffer pH 11.5/ACN (30:70) solution as the mobile phase, an analyzing voltage of 10 kV, and a temperature of 20°C. Applying these conditions on a Chiralpak AD-RH column, an analysis time of 7 min was needed to complete a separation, which was approximately half compared with a published LC method.

The WLS calibration curve using the area ratios (impurity/internal standard) results in predicted concentrations between 88 and 118% when spiked bulk- and formulation samples were used. For the calibration curve obtained using only the areas of the *R*-enantiomer, good linearity was also observed with predictions between 84 and 118%. Repeatability of injection was demonstrated, as RSD values of 0.68, 2.94, 4.28, and 9.30% were achieved for retention time, resolution, ratio, and area of the *R*-enantiomer, respectively. When repeatability and between-days variability were compared, the RSD values of the latter are higher, which was expected. These experiments also indicated that it is better to make a daily calibration curve. Summarized, the developed method is applicable to quantify the enantiomeric impurity in concentrations starting from 0.08% with respect to the active enantiomer in a relatively accurate manner.

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