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Use of a Hepta-Tyr antibiotic modified silica stationary phase for the enantiomeric resolution of D,L-loxiglumide by electrochromatography and nano-liquid chromatography

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Abstract

Hepta-Tyr antibiotic modified silica stationary phase was used for the chiral resolution of D,L-loxiglumide, a new drug under investigation proposed for the treatment of gastrointestinal diseases. The chiral stationary phase was packed into fused silica capillaries of 75 μm i.d. for a length of only 7 cm and used for both capillary electrochromatography (CEC) and nano-liquid chromatography (nano-LC) running the experiments with the same instrumentation; in order to increase the electroosmotic flow (EOF) the antibiotic stationary phase was mixed with amino-silica particles (3:1, w/w) generating a relatively high reversed EOF. The enantiomeric resolution of loxiglumide by CEC was strongly influenced by several experimental parameters such as applied electric field, mobile phase composition, capillary temperature, etc. Optimum experimental conditions were found applying 15 kV at 20 °C and eluting with acetonitrile–sodium phosphate buffer at pH 6 (1:1, v/v). The same capillary was tested for nano-LC experiments. Good chiral separation of loxiglumide was achieved selecting the appropriate mobile phase considering the type and concentration of organic modifier. The nano-LC optimised method was therefore validated and applied to the analysis of a pharmaceutical formulation declared to contain only D-loxiglumide.

Keywords: Electrochromatography; Enantiomer separation; Stationary phases, electrochromatography; Stationary phases, LC; Loxiglumide

1. Introduction

The separation of enantiomeric compounds is an important topic of research in analytical chemistry, especially in the pharmaceutical field where very often one of the enantiomer of a certain drug can exhibit very different pharmacological/biological activity. Due to the importance of the impact that unwanted enantiomers can have with people's health, there is a need of analytical methods for the quantitation of chiral compounds for, e.g., chiral purity control, pharmacokinetic studies [1].

A wide number of investigations concerns the development of analytical methods in order to perform enantiomeric analysis rapidly, at low costs, with high efficiency and high resolution.

Analytical methods so far employed for chiral drug resolution include high performance liquid chromatography (HPLC) [2–4], gas chromatography (GC) [5], thin-layer chromatography (TLC) [6], capillary electrophoresis (CE) [7–9] and recently capillary liquid chromatography (cLC)/nano-liquid chromatography (nano-LC) [10–13].

Capillary electrochromatography (CEC), one of the CE modes, is a powerful tool for chiral resolution offering separations in short time, high resolution and high efficiency utilizing advantages of both CE and HPLC, e.g. high efficiency and selectivity, respectively [9,14].

The use of miniaturized techniques (CEC and nano-LC) can offer several advantages over other conventional ones such as HPLC, e.g., increased mass sensitivity, higher efficiency, possibility of packing particles of lower diameter, lower consumption of mobile phase, more easy coupling with mass spectrometry (MS).

The separation of enantiomeric compounds is based on differences in affinity for the chiral selector used that can be

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either bonded to the capillary wall or to the silica or to polymeric material (open tubular, packed or monolithic material) [15].

Several chiral selectors were used for enantiomeric resolutions employing CEC and among them glycopeptidic antibiotics (GAs) such as vancomycin, teicoplanin, Hepta-Tyr seem to be powerful enantiorecognition agents towards a wide number of compounds [9,15,16].

The Hepta-Tyr antibiotic, belonging to the teicoplanin family was firstly used by us in capillary zone electrophoresis (CZE) for the enantiomeric resolution of several acidic compounds such as mandelic acid derivatives and herbicides [17,18] and recently this chiral selector was also applied to CEC for fast chiral resolution of hydroxy acid compounds achieving good resolution in less than 72 s [19].

(D,L)-4-(3,4-Dichlorobenzamido)-5-[*N*-(3-methoxypropyl)-*N*-pentylamino]-5-oxopentanoic acid, CR2017 also called loxiglumide is a selective antagonist and potent receptor of the cholecistokinase 1 (CCK-A). This compound was recently introduced in order to treat gastrointestinal pathologies and therefore due to its superior potency, the (D)-enantiomeric form is now under clinical investigation.

In this study, we investigated the enantioseparation of loxiglumide by using CEC and nano-LC employing the same capillary packed with a short length of chiral stationary phase (7.0 cm). In order to optimize the enantiomeric separation of loxiglumide we studied the effect of several experimental parameters such as mobile phase composition (organic modifier type, concentration ratio), capillary temperature, buffer concentration on chiral resolution, retention time, retention factor and efficiency.

The nano-LC method was validated and evaluated for the analysis of a pharmaceutical formulation declared to contain only D-loxiglumide.

2. Experimental

2.1. Instrumentation

CEC experiments were performed in a Agilent 3D CE instrument (Waldbronn, Germany) equipped with a UV-diode array detector operated at 206 nm (unless otherwise stated) and a thermostated capillary cartridge applying different voltages in the range 2.5–30 kV. Injection was done at the short end of the capillary (cathodic polarity) applying 12 bar × 0.2 min. During the experiments both ends of the capillary were pressurized at 8 bar in order to avoid bubble formation.

The nano-LC enantiomeric separations were carried out with the same instrument used for CEC analysis injecting the samples at the short end of the capillary and eluting mobile phase and analytes in an isocratic mode at 12 bar. The flow rate was not measured due to the configuration of the commercial instrumentation used that did not allow the acquisition of such data.

Fused silica capillaries (75 μm i.d. × 375 μm o.d.) were purchased from Composite Metal Services (Hallow, UK). Capillary packing was done by using a LC series 10 HPLC pump (Perkin-Elmer, Palo Alto, CA, USA).

2.2. Chemicals

Racemic loxiglumide, D- and L-loxiglumide and the pharmaceutical preparation containing the pure enantiomer D-loxiglumide were kindly supplied by Rotta Research Lab. (Monza, Italy). Phosphoric acid (85%), sodium hydroxide, acetonitrile (MeCN), methanol (MeOH) were purchased from Carlo Erba (Milan, Italy). Sodium cyanoborohydride, sodium periodate, LiChrospher diol silica phase 5 μm particle diameter (pore size 100 Å) and LiChrospher silica (Si-60, 5 μm) were purchased from Merck (Darmstadt, Germany) while Kromasil amino silica 5 μm (pore size 100 Å) was a gift of Aka Chemicals (Bohus, Sweden). Hepta-Tyr antibiotic (MDL 63,246) was kindly supplied by Professor P.G. Righetti, University of Verona, Italy. The synthesis of the chiral stationary phase was previously described [19].

2.3. Preparation of packed capillaries

The packing procedure of the fused silica capillaries used in this work was very similar to that previously described [19] with the following modification: frits were prepared using LiChrospher silica suspended in 5 mM of sodium chloride with a heated wire at about 600 °C for 8 s, the total length was 33.0 cm.

3. Results and discussion

3.1. Separation of loxiglumide enantiomers by using capillary electrochromatography

Based on our experience and considering the chemical structure of the studied analytes (see Fig. 1) for our experiments we selected Hepta-Tyr antibiotic (MDL 63,246) because the chiral selector exhibited very high recognition capability towards several acidic enantiomeric compounds in both CEC and nano-LC [17–19]. Preliminary experiments by CEC employing the packed capillary with silica modified Hepta-Tyr did not allow the analysis of loxiglumide

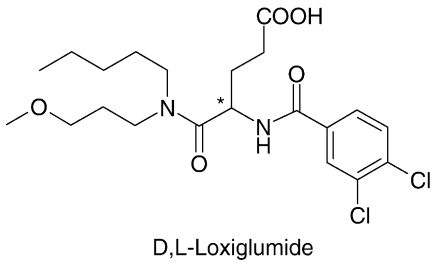

Fig. 1. Chemical structure of loxiglumide.

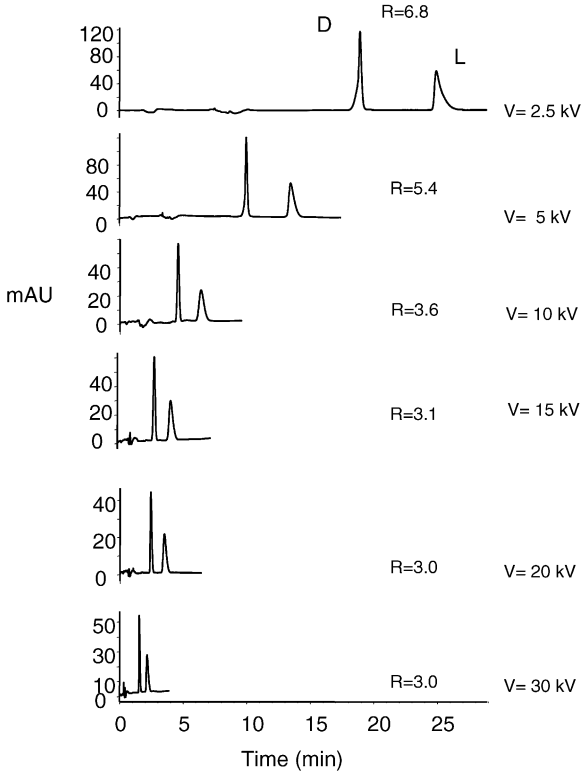

Fig. 2. Effect of applied voltage on retention time and resolution of loxiglumide enantiomers by using CEC. Mobile phase, 50% acetonitrile and 50% aqueous sodium phosphate pH 6 (final concentration, 5 mM); capillary 75 μm × 33 cm (effective length, 8.4 cm; packed, 7 cm); pressurized both sides at 8 bar; electrokinetic injection, 10 kV, 5 s of 0.2 mg/mL *rac*-loxiglumide; applied voltage, 2.5–30 kV.

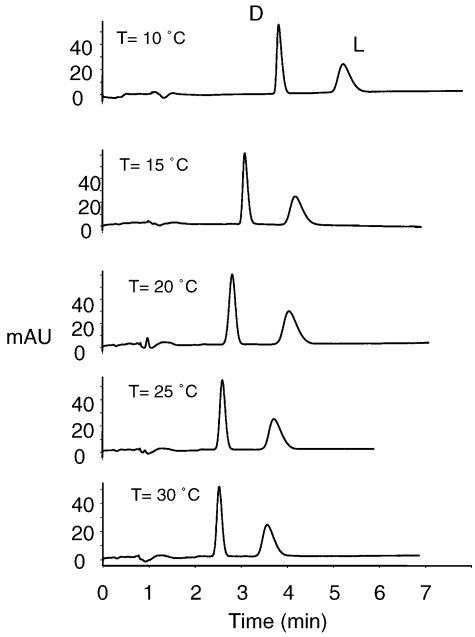

Fig. 3. Effect of capillary temperature on enantiomeric separation of loxiglumide enantiomers. Applied voltage, −15 kV, 4.4–5.5 μA. For other experimental conditions, see Fig. 2.

enantiomers because a too low electroosmotic flow (reversed to anodic direction) and unstable current. Therefore the capillary was packed with a mixture of silica based Hepta-Tyr and amino-silica particles (3:1, w/w). The used ratio (chiral stationary phase/amino-silica) and the CEC run performed at the short effective length of the capillary [19] were the appropriate compromise in order to achieve good enantioresolution in a relatively short time. The racemic mixture of loxiglumide was analyzed, after electrokinetic injection, employing a mobile phase of acetonitrile–5 mM sodium phosphate pH 6 (50:50, v/v). The relative standard deviation for retention time and resolution was satisfactory (2.0–2.2% for t_R and 2.9% for R_s; $n = 9$).

The effect of different applied electric fields on retention time and resolution of the two loxiglumide enantiomers was studied running the experiments in the range 75.8–909.1 V/cm (2.5–30 kV). As can be observed in Fig. 2, increasing the applied electric field, both retention times and resolution decreased. Further experiments were carried out applying 15 kV that allowed a good enantioresolution in a relatively short time with a reasonable current (4.7 μA).

The capillary temperature is an important parameter to control in order to optimize the CEC method because it can have strong influence on viscosity and on the kinetics of mass transfer. Considering the method employed in this study where the two enantiomers are transported to the detector by a strong electroosmotic flow associated with the electrophoretic mobility of the two analytes we observed the influence of capillary temperature on loxiglumide enantioresolution (see Fig. 3). As can be seen (perturbance of the baseline at 1.0–1.5 min) a slight increase of EOF was associated with a remarkable reduction of retention time thermostating from 10 to 30 °C. Enantioselectivity did not change markedly by increasing the capillary temperature, while retention factor increased reaching a maximum at 20 °C (e.g., $k_D = 1.88$) and slightly decreasing at 25 and 30 °C (1.80 and 1.79, respectively) (results not shown). The changes of capillary temperature did not affect the efficiency of the second eluted enantiomer while decreased by increasing the value of this parameter for the D-loxiglumide; similar trend was observed for enantioresolution (see Fig. 4a and b).

The content of the organic solvent present in the mobile phase was modified in the range 40–70%. Fig. 5 shows the effect of acetonitrile present in the mobile phase on enantioseparation of analytes by CEC. Raising the MeCN concentration we observed a decrease of retention time and resolution factor. The enantioselectivity firstly increased passing from 40 to 50% of MeCN and then decreased. The data showed that replacing water with an organic modifier in the mobile phase is causing a decrease of affinity of analytes towards the whole stationary phase. The repeatability of the CEC method was verified analysing six times a racemic mixture of loxiglumide running the experiments with the mobile phase containing 50% of MeCN and sodium phosphate pH 6 (5 mM final concentration). R.S.D.s of 1–1.5% were found for retention time

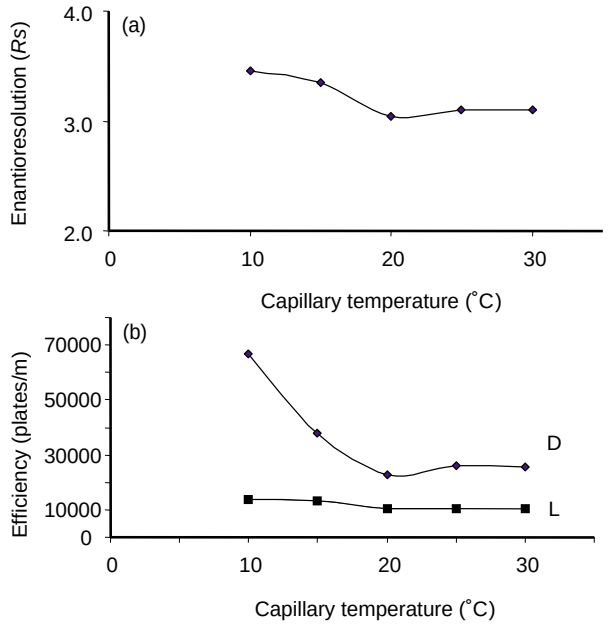

Fig. 4. Effect of capillary temperature on: (a) enantioresolution and (b) efficiency of studied loxiglumide enantiomers by CEC. For experimental conditions, see Figs. 2 and 3.

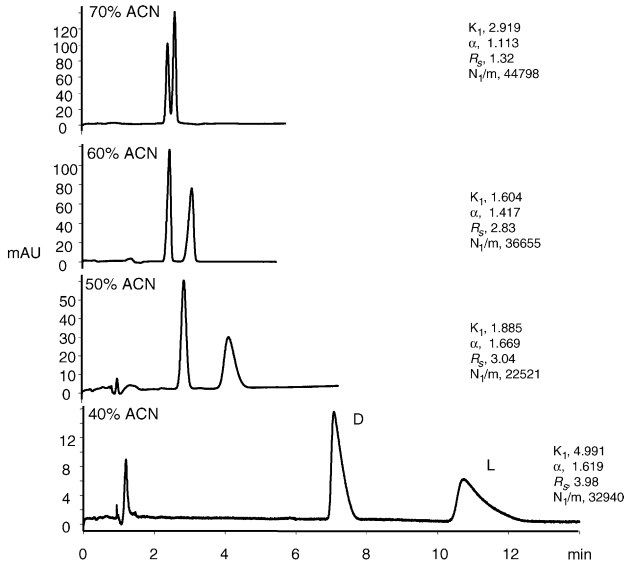

Fig. 5. Effect of acetonitrile concentration in the mobile phase on CEC enantioseparation of loxiglumide. For experimental conditions, see Figs. 2, 3 and text.

while for peak area less satisfactory results were achieved (2–3.5%).

3.2. Enantiomeric separation of loxiglumide by using nano-liquid chromatography

The same capillary used in CEC for the enantiomeric resolution of loxiglumide, packed with Hepta-Tyr antibiotic as the chiral selector and aminosilica (3:1, w/w), was employed in nano-liquid chromatography running the experiments with the same electrophoresis instrumentation. Due to the characteristics of the apparatus used, the pressure that we could apply was relatively low (12 bar) that is not useful when employing packed columns (23 cm or more) because long analysis time is expected. However, in our study, the short packed column and the high enantioselectivity were effective in achieving a good enantiomeric resolution of the studied analytes in a relatively short time.

In order to optimize the nano-LC method we investigated the effect of several experimental parameters such as mobile phase composition (organic modifier type and concentration), capillary temperature on retention time (t_R), resolution (R_s), retention factor (k) and number of theoretical plates (N/m).

The mobile phase was a mixture of sodium phosphate buffer at pH 6 (100 mM) and acetonitrile where in each experiment the final concentration of the buffer was 5 mM.

The effect of MeCN concentration present in the mobile phase used for the elution of loxiglumide enantiomers employing nano-LC is shown in Table 1. From these data it can be observed that the t_0 and retention times of the two enantiomers decreased by raising the MeCN concentration; similar results were observed for enantioresolution that decreased by increasing the organic modifier concentration. While efficiency (observed for the first enantiomer) increased from 40 to 60% of MeCN and then decreased. The highest retention factors were achieved at the lowest concentration of MeCN, these parameters decreased by increasing the MeCN concentration up to 60% and then increased again. As an example of the good performance of the nano-LC system, Fig. 6 reports the enantiomeric separation of D,L-loxiglumide using the mobile phase with 50% of acetonitrile that allowed a baseline chiral resolution of studied compounds in less than 5 min with good efficiency.

This mobile phase was further investigated keeping constant the content of organic modifier (50%, v/v) and introducing methanol; therefore the organic modifier present in the mobile phase was 50% of MeOH–MeCN where the ratio of the two solvents was changed from 0 to 1 (MeOH/MeCN). The increase of MeOH concentration caused an increase of the retention time of both enantiomers: the chiral resolution of loxiglumide was achieved in less than 5 and 35 min when the mobile phase contained 50% of MeCN and MeOH,

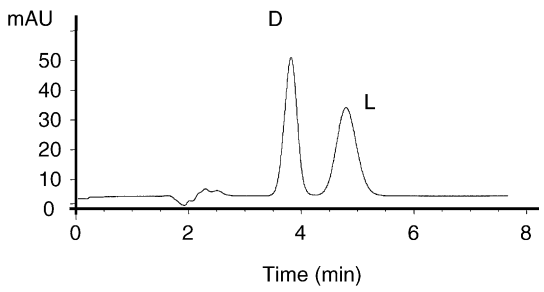

Fig. 6. Chromatogram of the enantiomeric separation of loxiglumide by using nano-liquid chromatography. Capillary and mobile phase used as described in Fig. 2; injection, hydrodynamic at 12 bar, 0.2 min.

Table 1
Effect of acetonitrile concentration in the mobile phase on the loxiglumide enantiomeric resolution by nano-liquid chromatography

MeCN (%)	t_0	t_1	t_2	k_1	k_2	R_s	μ	N_1 (plates/m)
40	2.17	6.57	9.13	2.03	3.21	2.25	1.58	11,738
50	1.93	3.85	4.85	1.00	1.51	1.74	1.52	14,036
60	1.77	2.88	3.27	0.63	0.85	1.07	1.35	14,679
70	1.59	2.84	3.32	0.79	1.10	0.91	1.38	6,024

Mobile phase, variable concentrations of acetonitrile–aqueous phosphate buffer pH 6 (with final concentration 5 mM); capillary as reported in Fig. 2.

respectively, while the t_0 did not change. These data clearly show that the two analytes were strongly interacting with the stationary phase and the process was enormously influenced by the type of organic modifier. This is also documented by Fig. 7a and b where the effect of the organic modifier type and concentration in the mobile phase on enantioselectivity and ln k was studied. α and ln k increased by raising the MeOH concentration. The enantioresolution of the studied analyte increased almost linearly by increasing the MeOH concentration and decreasing that of MeCN (R = 2.37–4.76). This is due to the different properties of the two organic solvents, e.g., polarity, H-bonding (donor/acceptor-character).

The efficiency (number of theoretical plates/m) for the first eluted enantiomer (D-loxiglumide) was in the range 14,800–24,095. The value of N/m increased by increasing the MeOH concentration reaching a maximum at 20% of alcohol and then decreased. The study of the capillary temperature did not show remarkable variation of the observed parameters.

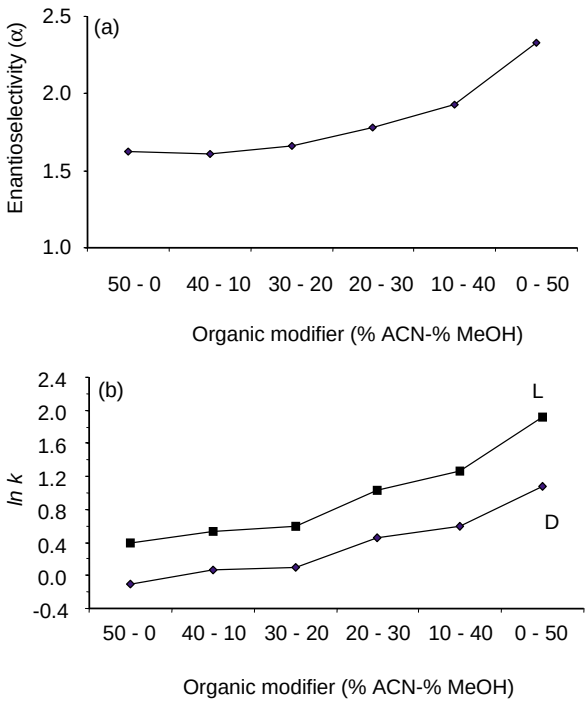

Fig. 7. Effect of organic modifier type and ratio on: (a) enantioselectivity and (b) ln k. For experimental conditions, see Figs. 2 and 6.

4. Validation of the nano-LC method

Validation of the method was performed using 0.2 mg/mL as racemic loxiglumide test concentration, 206 nm as detection wavelength eluting with 5 mM sodium phosphate pH 6–acetonitrile (1:1, v/v) at 20 °C, experimental conditions that allowed to achieve the best results concerning enantiomeric resolution and short analysis time.

4.1. Selectivity

In order to assess the selectivity of the method, the loxiglumide racemic mixture was analyzed and the baseline separation of the two enantiomers achieved with an average resolution factor R_s = 1.76 (n = 10, R.S.D. = 0.6%). The elution order of the two resolved enantiomers was verified by analysing a mixture D/L loxiglumide (3:1, v/v) observing that the D-isomer is firstly detected, clearly showing that the L-loxiglumide is showing an higher affinity towards the chiral selector present into the packed capillary.

4.2. Retention time, peak area repeatability and reproducibility

The standard mixture containing racemic loxiglumide was analysed by nano-LC 10 times during the day and in order to verify the repeatability (within-day precision) of the method. The same mixture was analysed over eighth days to evaluate intermediate precision (between-day precision). The obtained results are reported in Table 2.

Three capillaries of the same i.d. were packed as previously described and tested in order to verify the reproducibility analyzing the racemic mixture of loxiglumide. The experiments showed a R.S.D. lower then 5% for retention times while for peak areas was 7%.

4.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined from three and ten times the signal-to-noise ratio (S/N) values, where the noise was calculated by Agilent Technologies ChemStation software. Good sensitivity was obtained for both enantiomers finding LOD as low as 0.5 and 0.3 μg/mL for L- and D-loxiglumide, respectively while LOQ of 1.8 and 1.0 μg/mL for L- and D-isomer, respectively.

Table 2
Inter-day and intra-day repeatability for the enantiomeric separation of D,L-loxiglumide by using nano-liquid chromatography employing Hepta-Tyr antibiotic silica stationary phase

	Parameters							
	t_0 (min)	t_1 (min)	t_2 (min)	k_1	k_2	R_s	α	N_1/m
Intra-day								
Mean	1.89	3.70	4.68	0.96	1.48	1.76	1.54	13,827
R.S.D. (%) (n = 10)	0.9	1.2	1.4	1.0	1.2	0.6	0.3	1.2
Inter-day								
Mean	1.91	3.76	4.71	0.97	1.47	1.73	1.51	13,863
R.S.D. (%) (n = 8)	0.8	1.5	2.5	1.6	4.2	1.2	3.9	1.4

4.4. Linearity and accuracy

The linearity of the optimised method was verified for both enantiomers and in the selection of the concentration range it was considered that the L-isomer could be present at trace levels. Therefore, the calibration graph was linear in the range: 0.15–0.45 mg/mL (50–150% of the test concentration) and 0.005–0.030 mg/mL for D- and L-loxiglumide, respectively. The correlation coefficients found were: R^2 = 0.9984 and 0.9985 for D- and L-loxiglumide, respectively.

The accuracy was evaluated for the D-isomer at three concentration levels covering the linearity range obtaining (n = 3, $\alpha/2$ = 0.025) 101.97 ± 3.34, 101.30 ± 2.49, 99.88 ± 2.86 at concentration levels of 0.16, 0.30, 0.44 mg/mL, respectively.

4.5. Analysis of the pharmaceutical preparation

The optimized method was applied to the assay of a pharmaceutical sample (ampoule) declared to contain only D-loxiglumide (0.5%, w/v)

The analysis was performed in quadruplicate and the recovery of D-loxiglumide was in agreement with the labelled content ($\alpha/2$ = 0.025, recovery = 99.88 ± 2.16%, R.S.D. = 2.16%). The L-loxiglumide was not detected in the pharmaceutical sample because absent or at concentration lower than the LOD value.

5. Conclusions

From the above discussed results we can conclude that the use of a capillary packed with silica stationary phase modified with Hepta-Tyr antibiotic can be useful for the enantiomeric resolution of racemic loxiglumide using both electrochromatography and liquid chromatography in capillary format. Baseline chiral resolution was achieved with both techniques keeping advantages of nanofluidic methods where minute volumes of mobile phases are employed saving money and reducing pollution. Comparing the two methods, CEC exhibited higher enantioresolution as well higher efficiency than CLC, however for practical analysis we preferred to use nano-LC because the application of the electric field to the stationary phase requested longer time for capillary equilibration.

Acknowledgments

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References

[1] R. Innes, M. Nickersen, L.S. Goodman, A. Gilman (Eds.), The Pharmacological Basis of Therapeutics, MacMillan, New York, 1970, p. 477.
[2] C.P. Granville, B. Gehrcke, W.A. Konig, I.W. Wainer, J. Chromatogr. 622 (1993) 21.
[3] N.M. Maicr, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[4] F. Gasparrini, I. D'Acquarica, M. Pierini, C. Villani, J. Sep. Sci. 24 (2001) 941.
[5] V. Schurig, J. Chromatogr. A 906 (2001) 275.
[6] H.Y. Aboul-Enein, M.I. El-Awady, C.M. Heard, J. Pharm. Biomed. Anal. 32 (2003) 1055.
[7] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, New York, Weinheim, 1997.
[8] S. Fanali, J. Chromatogr. A 875 (2000) 275.
[9] S. Fanali, P. Catarcini, G. Blaschke, B. Chankvetadze, Electrophoresis 22 (2001) 3131.
[10] L. Chankvetadze, I. Kartozia, C. Yamamoto, B. Chankvetadze, G. Blaschke, Y. Okamoto, Electrophoresis 23 (2002) 486.
[11] Z. Chen, K. Uchiyama, T. Hobo, J. Chromatogr. A 942 (2002) 83.
[12] Z. Liu, K. Otsuka, S. Terabe, M. Motokawa, N. Tanaka, Electrophoresis 23 (2002) 2973.
[13] X. Chen, H. Zou, Z. Zhang, Electrophoresis 23 (2002) 1246.
[14] H. Hoffstetter, O. Hoffstetter, V. Schurig, J. Microcol. Sep. 10 (1998) 287.
[15] S. Eeltinik, G.P. Rozing, W.Th. Kok, Electrophoresis 24 (2003) 3935.
[16] B. Chankvetadze, J. Sep. Sci. 24 (2001) 691.
[17] S. Fanali, Z. Aturki, C. Desiderio, A. Bossi, P.G. Righetti, Electrophoresis 19 (1998) 1742.
[18] S. Fanali, Z. Aturki, C. Desiderio, P.G. Righetti, J. Chromatogr. A 838 (1999) 223.
[19] S. Fanali, P. Catarcini, C. Presutti, R. Stancanelli, M.G. Quaglia, J. Chromatogr. A 990 (2003) 143.