Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 842–852

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Separation and characterization of modified pregabalins in terms of cyclodextrin complexation, using capillary electrophoresis and nuclear magnetic resonance

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ARTICLE INFO

Article history:
Received 19 August 2009
Received in revised form 7 October 2009
Accepted 9 October 2009
Available online 21 October 2009

Keywords:
Enantioseparation
Synthesis
Lyrica
Capillary electrophoresis
NMR

ABSTRACT

The (*S*)-(+)-isomer of 3-isobutyl-GABA (pregabalin), the blockbuster drug in the treatment of neuropathic pain has been separated from its *R* isomer by cyclodextrin modified capillary zone electrophoresis (CZE) using uncoated fused-silica capillary. Derivatization of the single isomer and the racemate with tosyl- and dansyl-chloride was carried out to introduce strong UV chromophores of different size. CE-pH titrations were performed to determine the dissociation constants for both derivatives. 30 cyclodextrin (CD) derivatives as chiral agents were used at four different pH values to study the enantioseparation of the differently protonated guest molecules. The separation was optimized as a function of CD concentration, buffer type and concentration, pH and applied voltage. For the tosylated derivate the best resolution (R_s = 2.76) was found with 6-monodeoxy-6-mono-(3-hydroxy)-propylamino-beta-cyclodextrin hydrochloride (PA-β-CD) at pH 6.8, while with the same selector at pH 7.2 enantioseparation with an R_s value of 4.32 could be achieved for the dansylated pregabalin. At pH 2.5 for the dansylated derivative trimethylated alpha- and beta-CD systems resulted the most significant separation (R_s = 7.38 and R_s = 7.74, respectively). Experiments with dual CD systems were carried out as well. The stoichiometry of the complexes was determined using the Job plot method and resulted in a 1:1 complex in both cases. The structures of the inclusion complexes were elucidated using 2D ROESY NMR experiments.

1. Introduction

Enantiomeric purity is a key issue of chiral drugs for quality control and biological activity. Pharmacopoeias require sensitive, selective, fast, robust, inexpensive and environment-friendly methods to control the chiral purity [1,2]. Beside other separation techniques, such as high-performance liquid chromatography and gas chromatography, capillary electrophoresis (CE) eminently meets all these requirements. CE has become a powerful tool in the field of chiral analyses as it provides a rapid method development [3]. Additional advantage is that trace enantiomer impurities below 0.1% can even be routinely determined due to its high separation efficiency. Among the various chiral selectors, cyclodextrins represent the major class of chiral applications to optimize the separation of two enantiomers in capillary electrophoresis via complexation [4]. CE is suitable for the investigation of both stability constants and enantioseparating properties of CDs with various guest molecules.

CDs are neutral glucose oligomers of truncated cone shape, and possess a hydrophilic exterior surface and a hydrophobic interior cavity, enabling them to form host–guest inclusion complexes with a wide range of pharmaceuticals in aqueous solution [5].

Pregabalin, chemically known as (*S*)-3-aminomethyl-5-methylhexanoic acid (**1**) (Fig. 1), an alkylated single isomer analogue of the main inhibitory neurotransmitter GABA [6] is indicated for the treatment of neuropathic pain associated with diabetic peripheral neuropathy [7], post-herpetic neuralgia [8], adjunctive therapy for adult patients with partial onset seizures and fibromyalgia [9]. Pregabalin binds with high affinity to the alpha2-delta subunit protein of voltage-gated calcium channels in central nervous system tissues, thereby reducing the release of excitatory neurotransmitters. The *R*(−) enantiomer has been reported to be 10 times less active in biological assays, so *R*(−) represents an optical impurity [10].

Pregabalin has been determined in human plasma using LC–MS–MS [11]. Since pregabalin has very weak UV absorption, the inexpensive analysis of the drug requires derivatization for UV or fluorescence detection. The determination of pregabalin in the serum has been performed by HPLC using picryl sulfonic acid [12] or *o*-phthaldialdehyde [13] as derivatizing agent. Early studies reveal the need for chiral analyses of pregabalin intermediates

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doi:10.1016/j.jpba.2009.10.010

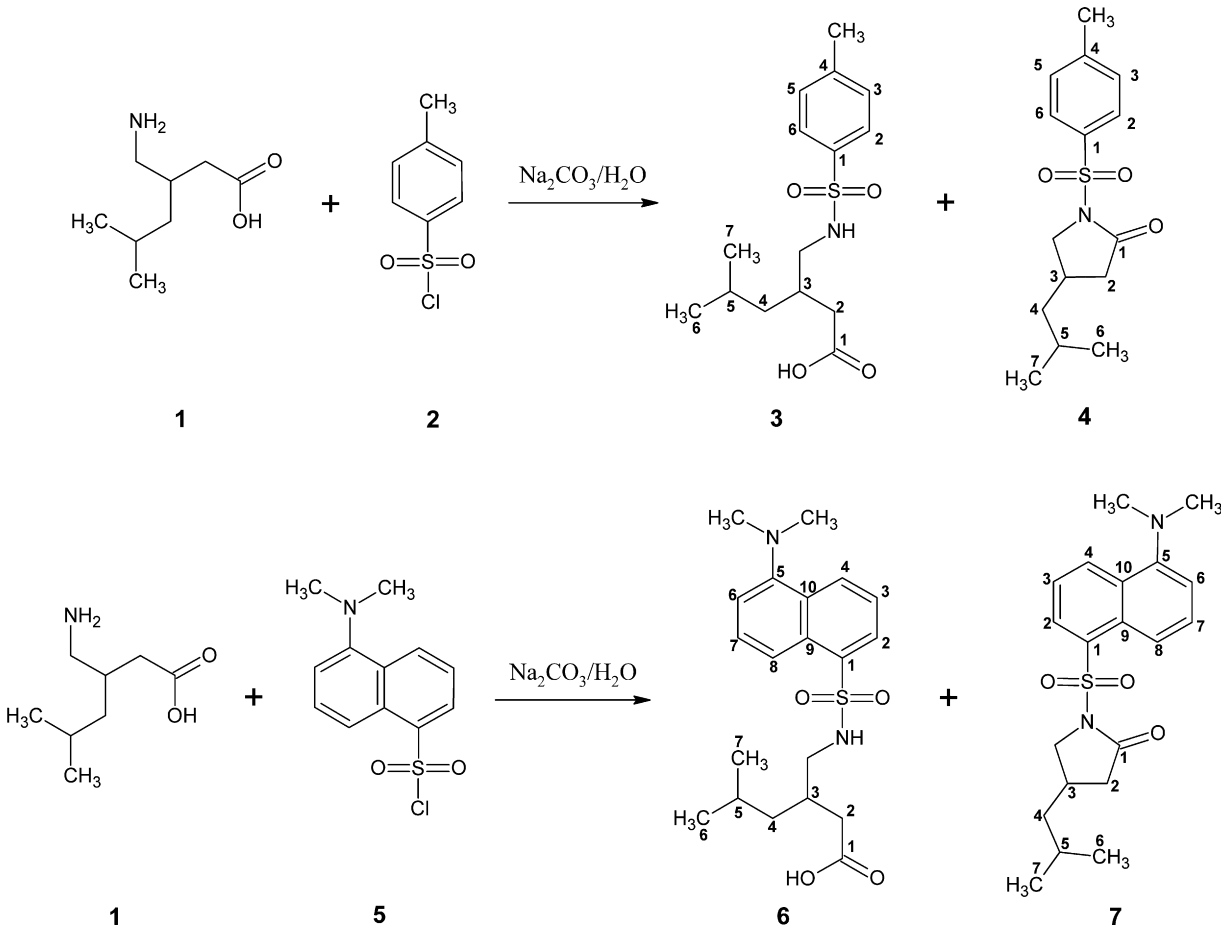

Fig. 1. Synthetic scheme, structure and numbering of the compounds studied. The systematic names are as follows **3**: 5-methyl-3-({[(4-methylphenyl)sulfonyl]amino}methyl) hexanoic acid **4**: 1-[(4-methylphenyl)sulfonyl]-4-(2-methylpropyl)pyrrolidin-2-one, **6**: 5-methyl-3-({[5-(dimethylamino)naphthalene-1-sulfonyl]amino}methyl)hexanoic acid **7**: 1-[5-(dimethylamino)naphthalene-1-sulfonyl]-4-(2-methylpropyl)pyrrolidin-2-one.

to determine their enantiomeric purities [14]. Three LC methods were mentioned in the literature for direct enantiomeric separation of pregabalin. One separation has been achieved using a macrocyclic glycopeptide stationary phase based, validated LC method with MS detection [15], while the other two methods were performed on ordinary C18 columns after derivatization of the racemate with *N*-5-fluoro-2,4-dinitrophenyl-5-L-alanine amide [16] and [17].

To the best of our knowledge, no CE method has so far been applied for the chiral separation of pregabalin isomers. Tosyl- and dansyl-chloride as proven derivatizing reagents for amino acids are especially advantageous in cyclodextrin modified capillary zone electrophoretic separations due to their strong interaction with the apolar cavity of cyclodextrins. Complexation mechanisms of dansyl moieties with various cyclodextrins have been studied in the literature [18].

The present paper reports direct capillary zone electrophoretic (CZE) methods for the enantioseparation of racemic tosylated and dansylated 3-aminomethyl-5-methylhexanoic acid derivatives using 30 different native and synthetically modified CDs. To achieve baseline-level chiral separation, the electrophoretic conditions were also optimized. The effects of separation circumstances, such as the composition, concentration and pH of the background electrolyte (BGE), concentration of chiral additives and the applied voltage were examined. Effectivity of dual CD systems was also extensively investigated. The migration order of the enantiomers was in all cases determined by spiking the racemic samples with the appropriate derivative of (*S*)-3-aminomethyl-5-methylhexanoic acid. The stoichiometry and the 3D structure of the complexes were confirmed by the Job's method and 2D ROESY NMR measurements, respectively.

2. Experimental

2.1. Materials

Racemic and (*S*)-3-aminomethyl-5-methylhexanoic acid were kindly provided by the Pharmaceutical Company EGIS. All native CDs and their derivatives were the products of Cyclolab Ltd. (Budapest, Hungary). H_3BO_3, H_3PO_4, NaH_2PO_4, CH_3COOH and NaOH used for the preparation of buffer solutions for CE were of analytical grade purchased from commercial suppliers. The NMR solvents D_2O (>99.8 atom% D) and $CDCl_3$ were from Sigma. As EOF marker in CE experiments DMSO from Reanal (Budapest, Hungary) was used. All reagents were used without further purification. Bidistilled Millipore water (specific resistance: 18.2 MΩ cm) was used throughout this study.

2.2. Synthetic procedures

The tosylation and dansylation of both (*S*)- and the racemic 3-(aminomethyl)-5-methylhexanoic acid (**1**) were performed by a modified literature procedure [19]: 3-(aminomethyl)-5-methylhexanoic acid (**1**) (77 mg, 0.48 mmol) and *p*-toluenesulfonyl chloride (Sigma) (**2**) (110 mg, 0.58 mmol, 1.2 equiv.) or 5-(dimethylamino)naphthalene-1-sulfonyl chloride (Sigma) (**5**)

Table 1
^{1}H and ^{13}C NMR assignments of the synthesized compounds in $CDCl_3$.

Moiety	No.	**3**			**4**		
		^{13}C	^{1}H	m, *J* (Hz), int.	^{13}C	^{1}H	m, *J* (Hz), int.
Pregabalin	1	178.4			173.6		
	2	37.3	2.34	m, 2H	39.7	2.08	dd, (17.1, 8.9), 1H
						2.53	dd, (17.1, 8.1), 1H
	3	33.7	2.11	m, 1H	30.8	2.43	m, 1H
	4	42.0	1.13	m, 2H	43.5	1.28	m, 2H
	5	25.8	1.55	m, 1H	26.7	1.55	m, 1H
	6	23.3	0.85	d, (6.6), 3H	23.15	0.88	d, (6.6), 3H
	7	23.1	0.84	d, (6.6), 3H	23.19	0.90	d, (6.6), 3H
	N-CH_2	47.5	2.84	m, 1H	53.4	3.40	dd, (9.7, 7.8), 1H
			2.97	m, 1H		4.04	dd, (9.7, 7.6), 1H
	NH		5.07	brs, 1H			
Tosyl	1	137.6			135.9		
	2 and 6	127.8	7.73	d, (8.2), 2H	128.7	7.92	d, (8.3), 2H
	3 and 5	130.4	7.29	d, (8.2), 2H	130.3	7.33	d, (8.3), 2H
	4	144.1			145.8		
	4-CH_3	22.2	2.40	s, 3H	22.4	2.44	s, 3H

Moiety	No.	**6**			**7**		
		^{13}C	^{1}H	m, *J* (Hz), int.	^{13}C	^{1}H	m, *J* (Hz), int.
Pregabalin	1	178.9			173.3		
	2	37.1	2.24	d, (6.4), 2H	39.8	2.08	dd, (16.9, 8.5), 1H
						2.50	dd, (16.9, 8.5), 1H
	3	33.4	1.94	m, 1H	30.4	2.42	m, 1H
	4	41.6	0.96	m, 1H	43.4	1.24	m, 1H
			1.02	m, 1H		1.29	m, 1H
	5	25.8	1.39	m, 1H	26.6	1.54	m, 1H
	6	23.1	0.73	d, (6.5), 3H	23.1	0.88	d, (6.6), 3H
	7	23.0	0.67	d, (6.5), 3H		0.86	d, (6.6), 3H
	N-CH_2	47.2	2.83	m, 1H	53.5	3.58	dd, (9.4, 7.8), 1H
			2.95	m, 1H		4.18	dd, (9.4, 7.8), 1H
	NH		5.38	bs, 1H			
Dansyl	1	135.3			134.0		
	2	130.3	8.22	dd, (7.3, 1.1), 1H	133.1	8.48	dd, (7.4, 1.3), 1H
	3	131.1	7.49	dd, (8.6, 7.3), 1H	124	7.58	dd, (8.5, 7.4), 1H
	4	123.9	8.53	dd, (8.6, <0.5), 1H	132.4	8.60	dd, (8.5, <0.5), 1H
	5	152.5			152.9		
	6	116.0	7.18	dd, (7.4, <0.5), 1H	115.6	7.19	dd, (7.5, <0.5), 1H
	7	129.1	7.54	dd, (8.6, 7.4), 1H	129.3	7.57	dd, (8.7, 7.5), 1H
	8	119.5	8.31	dd, (8.6, <0.5), 1H	118.9	8.20	dd, (8.7, <0.5), 1H
	9	130.3			132.4		
	10	130.5			129.3		
	N-$(CH_3)_2$	46.1	2.84	s, 6H	46.1	2.89	s, 6H

(156 mg, 0.58 mmol, 1.2 equiv.) were suspended in 3 ml 1 M Na_2CO_3 aqueous solution and stirred for 4 h at room temperature. The pH was adjusted to 2 with concentrated HCl, and then water (5 ml) was added. The mixture was extracted with dichloromethane (2× 15 ml), the organic layer was dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by column chromatography with a mixture of *n*-hexane and ethyl acetate (2:1) to get the desired tosylated pregabalin **3** (**Tos-preg**) (white solid, yield 41%, mp 77–78 °C) and dansylated pregabalin **6** (**Dns-preg**) (green oil, yield 30%) (Fig. 1). As undesired by-products, their ring-closed forms, the appropriate pyrrolidin-2-one derivatives **4** (off-white solid, yield 7%, mp 71–72 °C) and **7** (yellow solid, yield 17%, mp 117–121 °C) were also isolated and characterized. This cyclization reaction has also been reported by Hoekstra et al. [20]. ^{1}H and ^{13}C NMR data of the synthesized compounds are collected in Table 1.

2.3. NMR measurements

All NMR experiments were carried out on a 600 MHz Varian VNMRS spectrometer equipped with a dual 5-mm inverse-detection gradient (IDPFG) probehead. In case of structure identifications standard pulse sequences and processing routines available in VnmrJ 2.2C/Chempack 4.0 were used. ^{1}H and ^{13}C chemical shifts in $CDCl_3$ were referenced to internal TMS (δ = 0.000 ppm) or to the residual solvent signal unless otherwise stated. In all experiments, the probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The ROESY spectra were recorded on 5 mM β-CD/γ-CD/PA-β-CD, 5 mM derivatized pregabalin and 70 mM Na_2HPO_4 samples in D_2O, applying mixing times ranging from 350 to 100 ms during a spin-lock of 2.2 kHz. 256 increments were collected with 32 repetitions and the measured data matrix was processed as a matrix of 4K (F2) by 1K (F1) data points. Intermolecular NOEs between pregabalin and cyclodextrin protons directly involved in the host-guest interaction were detected as cross-peaks. Job's analysis was carried out using 5 mM tosylated or dansylated pregabalin derivative and 5 mM β-CD at pH 7.2 in phosphate buffer (in H_2O/D_2O 9/1). 16–128 scans (depending on the experiment) with a spectral window of 4800 Hz were collected into 32,000 data points, giving a digital resolution of 0.3 Hz/point. For H_2O signal suppression the presaturation sequence was used.

2.4. CE measurements

All CE experiments were performed on a ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany), equipped with a photodiode array detector and the Chemstation software for data handling. An untreated fused-silica capillary (50 μm id, 64.5 cm total, 56 cm effective length) was purchased from Agilent. Conditioning of new capillaries was conducted by flushing with 1 M NaOH for 30 min followed by 0.1 M NaOH and buffer for 60 min each. The temperature of the capillary cassette was set to 25 °C. During measurements at pH 9.2, pH 7.2 and pH 4.7 +30 kV voltage, while at pH 2.5 −25 kV voltage was applied, unless stated otherwise. UV detection was performed at 200, 215, 220, 225 and 230 nm and samples were run in triplicate. Along with the UV traces the current and the voltage were monitored. Prior to all runs the capillary was preconditioned by flushing with water (0.5 min), 0.1 M NaOH (1 min), water (1 min) and BGE (2 min). The samples were injected hydrodynamically (50 mbar, 4 s).

The running buffers were 80 mM borate at pH 9.2, 50 mM phosphate at pH 7.2, 50 mM acetate at pH 4.7 and 15 mM phosphate at pH 2.5. The BGE contained CDs at various concentrations (2–75 mM). The **Tos-preg** and **Dns-preg** stock solutions (1.6 and 2.0 mM, respectively) contained 20% (v/v) MeOH and 0.001% (v/v) DMSO, the latter served as EOF marker. During CE-pH experiments for BGE acetate and phosphate buffers or their mixtures were used depending on the desired pH (ranging from 1.99 to 5.99).

As primary response functions t_{EOF}, t_1 and t_2 migration times were recorded. Effective mobility values were calculated as:

$$\mu_{eff} = \frac{l_c l_d}{U} \cdot \left(\frac{1}{t} - \frac{1}{t_0}\right)$$

where l_c is the total length of the capillary, l_d is the length of the capillary to the detector, U is the applied voltage and t and t_0 are the peak appearance times of the analyte and the EOF, respectively [21].

The binding constants of the inclusion complexes were determined using x-reciprocal linearization method [22]. The resolution (R_s) was applied as the secondary response function when evaluating the performance of the separation system and was calculated as:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where w_1 and w_2 stand for the extrapolated peak width at the baseline.

3. Results and discussion

3.1. Capillary electrophoresis

3.1.1. CE-pH titration

The inclusion study of differently protonated forms of both pregabalin derivatives required (first) the determination of the acid dissociation constant. The titration was carried out with capillary electrophoresis in the pH range of 3.68 and 5.78 for **Tos-preg** and of 1.99 and 5.99 for **Dns-preg**. For the determination of pH values calibrated pH meter reading was used. Using the capillary parameters, the applied voltage and the migration times, the effective mobility values were calculated and plotted as a function of pH. The dissociation constants were determined with the OPIUM program [23]. The observed electrophoretic mobility at a particular pH is a weighted sum of individual mobilities of the unprotonated (μ_A) and protonated (μ_{HA}, charges omitted) form of the molecule, which can be rearranged to the master equation of pK_a determination by CE. A curve was fitted on the observed data points using the following equation:

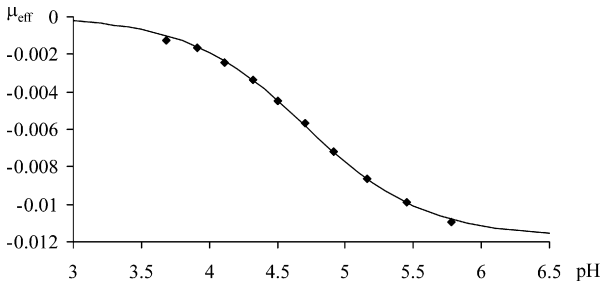

Fig. 2. CE-pH titration of Tos-preg along with the fitted curve.

$$\mu_{eff} = \frac{\mu_A + \mu_{HA} \cdot 10^{pK_a - pH}}{1 + 10^{pK_a - pH}}$$

Fig. 2 shows the CE-pH titration of **Tos-preg**, for which pK_a = 4.75 ± 0.02 was determined. The overlapping dissociation steps of the diprotic **Dns-preg** correspond to $pK_{a_1} = 3.47 \pm 0.06$ and $pK_{a_2} = 4.67 \pm 0.06$. The pH values applied in subsequent experiments were set upon these constants.

3.1.2. Enantioseparation

Due to Wren's theory, the optimal CD concentration for the enantioseparation can be calculated as:

$$[CD]^{opt}_{\Delta\mu} = \frac{1}{\sqrt{K_R K_S}}$$

where K_R and K_S are the stability constants of the inclusion complexes of the R and S enantiomer, respectively [24]. The crucial requirement of application of CE in binding analysis is that at least one of the interacting species has to carry a charge. For determination of the binding constants various concentration of CDs were added to the BGE at each pH value. Stability constants were determined with x-reciprocal method and the optimal selector concentration was then calculated by the Wren equation. The enantioseparation experiments were carried out at four different pHs to study the effect of protonation state on the complexation. Based on the pK_a values, both derivatives are in their most basic form and carry one negative charge at pH 9.2 and 7.2. At pH 4.7 they are still partially anionic, thus at these pH values all CDs are suitable for the CE analysis. As the **Tos-preg** is neutral at pH 2.5 the detection of enantiomeric separation is possible only with ionic CDs, while **Dns-preg** bears positive charge and was investigated with all selectors. The CDs applied in our study are listed in Table 2.

At pH 9.2, where the pregabalin derivatives bear negative charge for **Tos-preg** the HP-γ-, MA-β-, PA-β-, CE-β- and SP-γ-CDs, while for **Dns-preg** the HP-α-, HP-β- (DS ∼ 4.6), DIMEB-, MA-β-, PA-β- and CE-β-CDs gave enantiodiscrimination, although complex formation could be observed in almost all cases. Among these CD derivatives, PA-β-CD caused the most significant separation with both pregabalin derivatives, R_s = 1.45 for **Tos-preg** and R_s = 1.21 for **Dns-preg**. The effect of the PA-β-CD selector concentration on the enantioseparation of **Tos-preg** can be seen in Fig. 3A.

At pH 7.2 the BGE contained 50 mM phosphate buffer instead of 80 mM borate. As the guest molecules were still negatively charged, only the CDs that caused enantioseparation at pH 9.2 were investigated. The change in BGE resulted in a less effective separation in case of neutral (HP- and DIMEB-) and negative (CE-β- and SP-γ-) CDs. As at pH 7.2, MA-β- and PA-β-CDs are partially protonated, electrostatic interactions increase the stability of the inclusion complexes and improve the selectivity of the CDs. An additional experiment was carried out at this pH with PA-β-CD and **Dns-preg**

Table 2
Collection of tosyl- and dansyl-pregabalin enantioseparation data in CD- and pH-dependent systems, including abbreviations of the applied CDs, stability constants (K) and resolution (R_s).

Abbreviations	Name	Enantioseparation							
		Tos-preg				**Dns-preg**			
		pH 9.2	pH 7.2	pH 4.7	pH 2.5	pH 9.2	pH 7.2	pH 4.7	pH 2.5
α-CD	Alpha-cyclodextrin	– 63.8(1)		– 64(4)		– 51.6(6)		– 0	– 96(5)
β-CD	Beta-cyclodextrin	– 286(2)		– 252(19)		– 365.1(3)		– 195(11)	– 555(40)
γ-CD	Gamma-cyclodextrin	– 169(1)		+ S: 242(2) R: 230(2) R_s = 0.55		– 426(20)		+ S: 42.2(3) R: 34.1(2) R_s = 0.56	– 221(13)
HP-α-CD DS ~ 3[a]	(2-Hydroxy)propyl-α-CD	– 42(1)		– 55.4(7)		+ S: 43.7(5) R: 48.0(6) R_s = 0.40	– 28(1)	– 40(4)	– 38(4)
HP-β-CD DS ~ 3	(2-Hydroxy)propyl-β-CD	– 291(3)		+ S: 365(6) R: 363(6) R_s = 0.56		– 261(8)		– 252(6)	+ S: 470(40) R: 490(34) R_s = 1.05
HP-β-CD DS ~ 4.6	(2-Hydroxy)propyl-β-CD	– 265(2)		– 427(30)		+ S: 270(3) R: 309(4) R_s = 0.54	– 189(4)	– 141(6)	+ S: 182(7) R: 191(6) R_s = 1.16
HP-β-CD DS ~ 6.3	(2-Hydroxy)propyl-β-CD	– 177(5)		– 297(2)		– 414(1)		– 142(3)	+ S: 424(2) R: 426(2) R_s = 1.36
HP-γ-CD	(2-Hydroxy)propyl-γ-CD	+ S: 91(1) R: 82(1) R_s = 0.65	+ S: 57(5) R: 49(5) R_s = 0.54	+ S: 181(1) R: 165(1) R_s = 0.55		– 365(3)		+ S: 261(25) R: 218(19) R_s = 0.36	+ S: 154(6) R: 159(4) R_s = 0.39
TRIMEA-CD	Trimethylated-α-CD	– 201(10)		– 210(2)		– 86(2)		+ S: 109(3) R: 130(4) R_s = 2.30	+ S: 133(6) R: 125(5) **R_s = 7.38**
TRIMEB-CD	Trimethylated-β-CD	– 73(3)		– 16.8(6)		– 65(10)		+ S: 33.7(7) R: 52(1) R_s = 1.00	+ S: 104(5) R: 110(4) **R_s = 7.74**
TRIMEG-CD	Trimethylated-γ-CD	– 41(2)		+ S < 10 R < 10 R_s = 1.68		– 25(3)		– 0	+ S: 42(3) R: 40(4) R_s = 1.05

Table 2 (*Continued*)

Abbreviations	Name	Enantioseparation							
		Tos-preg				**Dns-preg**			
		pH 9.2	pH 7.2	pH 4.7	pH 2.5	pH 9.2	pH 7.2	pH 4.7	pH 2.5
DIMEB-CD 50[b]	Heptakis(2,6-di-O-methyl)-β-CD	–		–		+		–	+
DS ~ 14		202(9)		667(5)		S: 329(1) R: 268(1) R_s = 0.81		676(17)	S: 246(2) R: 251(2) R_s = 1.75
DIMEB-CD 98	Heptakis(2,6-di-O-methyl)-β-CD	–		–		+	+	–	+
DS ~ 14		249(6)		721(34)		S: 406(3) R: 270(1) R_s = 1.11	S: 147(2) R: 151(2) R_s = 0.57	893(75)	S: 360(2) R: 368(2) R_s = 3.87
RAMEB-CD DS ~ 12	Methylated-β-CD	– 248(1)		– 420(2)		– 325(18)		– 283(4)	+ S: 428(8) R: 436(6) R_s = 0.70
Ac-β-CD DS ~ 7	Acetylated-β-CD	– 505(7)		– 144(1)		– 527(3)		– 79(13)	– 198(8)
MA-β-CD	6-Monoamino-6-monodeoxy-β-CD	+	+	-	+	+	+	–	+
		S: 117(2) R: 116(2) R_s = 0.98	S: 361(1) R: 324(1) R_s = 1.72	0	S < 10 R < 10 R_s = 0.52	S: 70(2) R: 63(1) R_s = 1.03	S: 305(2) R: 204(1) R_s = 1.17	0	S < 10 R < 10 R_s = 0.59
PA-β-CD	6-Monodeoxy-6-mono(3-hydroxy)propylamino-β-cyclodextrin hydrochloride	+	+	–	+	+	+	–	+
		S: 277(1) R: 242(1) R_s = 1.45	S: 502(4) R: 358(4) R_s = 2.32 optimized **R_s = 2.76**	0	S < 10 R < 10 R_s = 1.19	S: 184(1) R: 141(1) R_s = 1.21	S: 399(5) R: 292(4) R_s = 2.85 optimized **R_s = 4.32**	0	S < 10 R < 10 R_s = 1.37
Succ-β-CD DS ~ 4	Succinylated-β-CD	– 0		– 29.7(8)		– 0		– 41(5)	– 231(17)
Succ-β-CD DS ~ 6	Succinylated-β-CD	– 0		– 29.3(8)		– 0		– 44(8)	– 298(10)
CM-β-CD DS ~ 1	Carboxymethylated-β-CD sodium salt	–		–		–		–	–
		0		0		0		0	0
CM-β-CD DS ~ 3	Carboxymethylated-β-CD sodium salt	–		–		–		–	–
		62(2)		0		85(1)		0	0
CE-β-CD DS ~ 3	Carboxyethylated-β-CD	+	–	–		+	–	–	+
		S: 29.7(3) R: 14.3(2) R_s = 0.87	47(2)	47(5)		S: 26.1(5) R: 10.4(2) R_s = 0.61	42(3)	71(5)	S: 336(2) R: 340(3) R_s = 1.15

SP-α-CD DS ~ 2	Sulfopropylated-α-CD sodium salt	–		–	+	–	–	–
		28(1)		0	S: 24(1) R: 27(1) R_s = 1.05	48(1)	0	0
SP-β-CD DS ~ 2	Sulfopropylated-β-CD sodium salt	–		–	+	–	–	+
		182(1)		36(8)	S: 288(2) R: 302(2) R_s = 1.02	128.0(4)	0	S: 329(12) R: 408(14) R_s = 1.00
SP-β-CD DS ~ 4	Sulfopropylated-β-CD sodium salt	–		–	+	–	–	–
		14.8(5)		0	S: 716(16) R: 760(18) R_s = 0.89	0	0	1286(11)
SP-γ-CD DS ~ 2	Sulfopropylated-γ-CD sodium salt	+	–	–	–	–	–	+
		S: 35(3) R: 38(3) R_s = 0.33	14.7(5)	17(2)	189(8)	90(2)	0	S: 29.5(2) R: 38.2(3) R_s = 1.38
P-β-CD DS ~ 4	Phosphated-β-CD sodium salt	–		–	+	–	–	+
		0		0	S: 680(20) R: 620(20) R_s = 1.16	0	0	S: 379(13) R: 368(12) R_s = 1.20
SHP-β-CD DS ~ 3	Sulfo(2-hydroxy)propylated-β-CD sodium salt	–		–	+	–	–	+
		17.8(8)		0	S: 376(15) R: 398(16) R_s = 0.98	22(1)	0	S: 110(3) R: 131(3) R_s = 1.83
SHP-γ-CD DS ~ 3	Sulfo(2-hydroxy)propylated-γ-CD sodium salt	–		–	–	-	–	+
		0		0	284(12)	67(3)	0	S: 73(2) R: 79(2) R_s = 1.10
SB-β-CD DS ~ 4	Sulfobutylated-β-CD sodium salt	–		–	+	–	–	+
		46(5)		0	S: 946(5) R: 1014(8) R_s = 1.92	0	0	S: 672(12) R: 756(15) R_s = 2.29

Uncertainties in parentheses are estimated standard deviations of the last significant digit (0 stands for indeterminable stability constants). Success of enantioseparation (+ or –) and resolution measured at optimal selector concentration according to Wren's formula at four pH values with both pregabalin derivatives.

[a] DS stands for degree of substitution, number of substituted hydroxyl groups per CD molecule.

[b] The number after the abbreviation means the isomer purity of the CD in %.

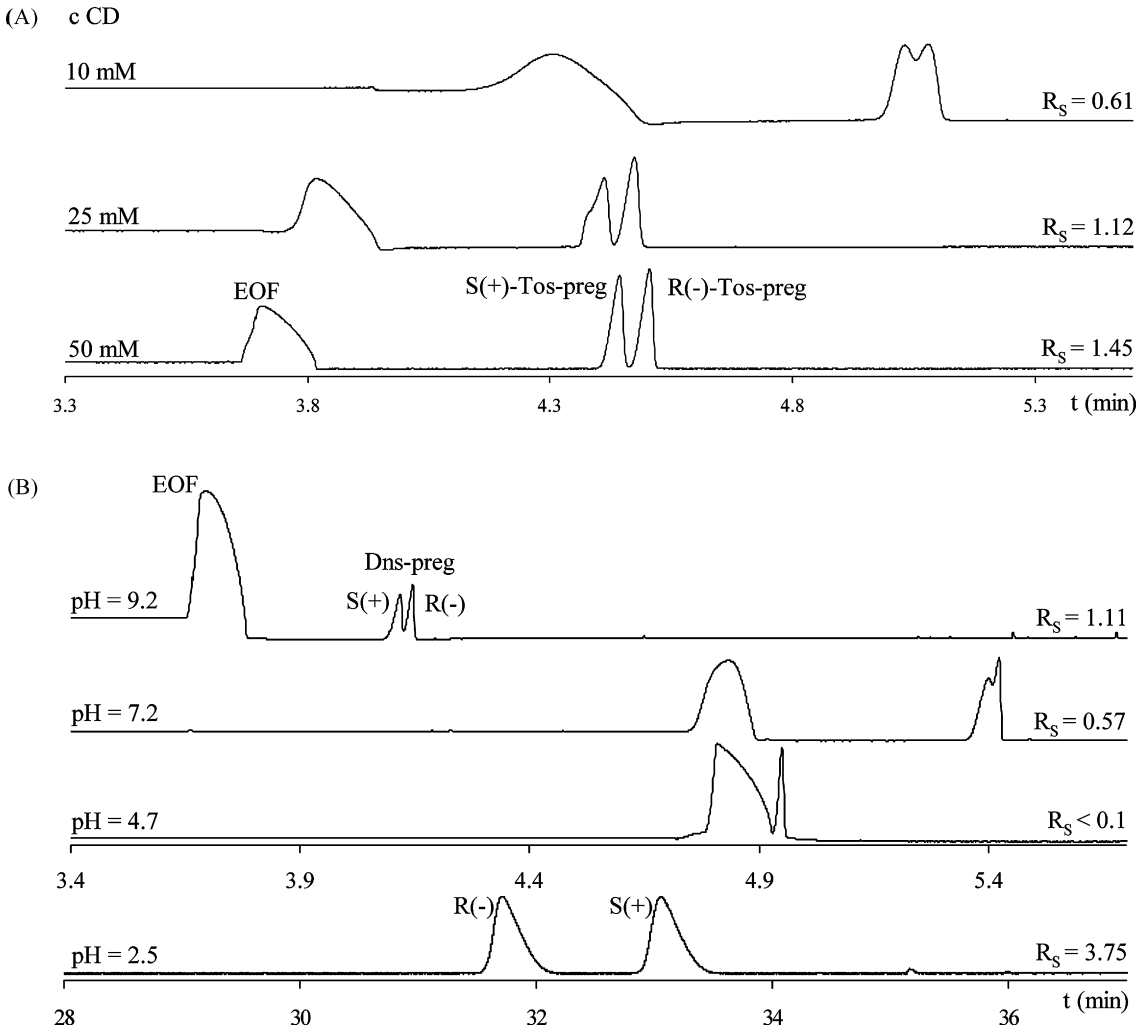

Fig. 3. (A) The enantioseparation of Tos-preg at various concentrations of PA-β-CD at pH 9.2. (B) The effect of pH on the resolution and migration order of Dns-preg enantiomers in DIMEB-CD systems at optimal CD concentration.

to compare the theoretical and empirical optimal CD concentration for the enantioseparation. The results were in excellent correlation (c_{theor} = 3.15 mM vs. c_{emp} = 3.2 mM), thus R_S determinations were carried out at the optimal concentration calculated by Wren's formula. The slight difference between the stability constants of the neutral CDs and the pregabalin derivatives can be observed at pH 9.2 and pH 7.2, though the charge of both the hosts' and the guests' are constant. This can be explained as the effect of the different composition and concentration of the BGE (80 mM borate vs. 50 mM phosphate).

At pH 4.7 50 mM acetate buffer was applied as BGE, where negatively charged and neutral pregabalin derivatives exist at commeasurable concentrations. For **Tos-preg** a partial separation with the γ-, HP-β- (DS ~ 3), HP-γ-CD and baseline separation with TRIMEG-CD could be achieved. Interestingly, TRIMEG-CD was the most effective chiral selector for **Tos-preg** (R_s = 1.68), despite its low stability constant (K < 10). In case of **Dns-preg** besides γ- and HP-γ-CD, TRIMEA- and TRIMEB-CD were the most suitable. Similarly to **Tos-preg**, the permethylated CD derivatives showed better enantioseparation.

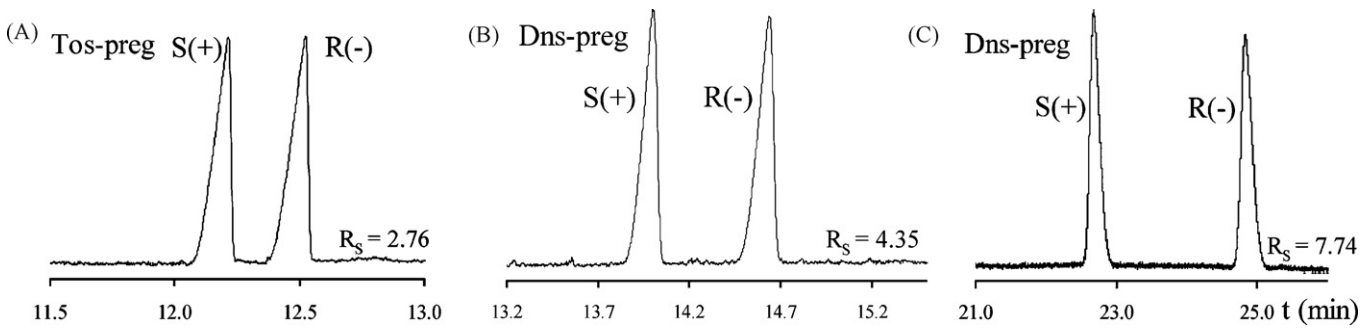

Fig. 4. The electropherograms of the optimized PA-β-CD systems (A and B) and the highest resolution achieved (pH = 2.5, 50 mM TRIMEB-CD) on the enantioseparation of **Dns-preg** (C).

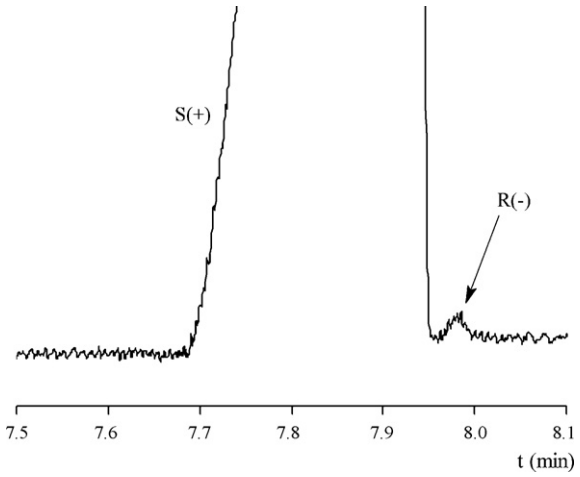

Fig. 5. Detection of R(−)-Tos-preg trace with the optimized PA-β-CD system in the presence of the single S(+) enantiomer at 1:1000 concentration ratio.

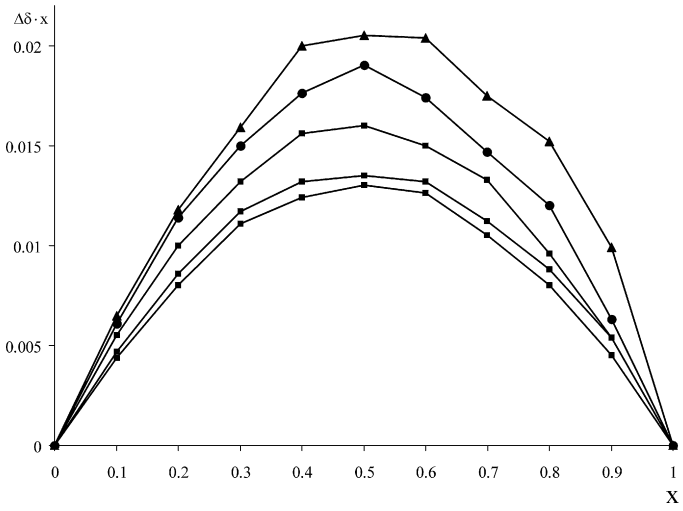

Fig. 7. Job's plot derived from the downfield changes in chemical shift of 2H, 3H, and 6H in Tos-preg's pregabalin moiety (square), 4H of Dns-preg's pregabalin moiety (triangle) and 3H β-CD protons (circle).

At pH 2.5, 15 mM phosphate buffer was used as BGE. As at this pH **Tos-preg** isomers migrate with the EOF, so their separation could be observed only with ionic CDs in CZE. However, Dns-preg is almost fully protonated, thus its complexation with all the applied CDs was investigated., All the applied CDs with the exception of SHP-γ- and SP-γ-CD gave partial or baseline enantioseparation in case of **Tos-preg**, the highest resolution however, was achieved with SB-β-CD (R_S = 1.92). A great number of CDs were applied successfully in the enantioseparation of **Dns-preg** enantiomers at pH 2.5. Among the ionic CDs, similarly to **Tos-preg** SB-β-CD caused pronounced enantioseparation (R_S = 2.29), while DIMEB, TRIMEA and TRIMEB as neutral CDs showed the most successful separation of all experiments (R_S = 3.87, R_S = 7.38, R_S = 7.74, respectively). Nevertheless, the optimal resolution of the di- and trimethylated CD systems can be achieved at high CD concentration (above 50 mM) turning the method uneconomical. An interesting effect of the size of CD cavity can be observed at pH 2.5 in case of trimethyled CDs resulting changes in migration order of **Dns-preg** enantiomers: with TRIMEA- and TRIMEG-CD the S(+) isomer, while with TRIMEB-CD R(−) migrates first. The binding constants for both enantiomers and resolution values of the above mentioned systems are shown at each pH in Table 2, the highest resolution values are highlighted in bold. The effect of pH on the enantioseparation and migration order of **Dns-preg** isomers with DIMEB-CD is depicted in Fig. 3B.

As Table 2 shows, the only system that caused major enantioseparation with both pregabalin derivatives was PA-β-CD at pH 7.2, these systems were therefore further optimized investigating the effect of smaller changes in pH, concentration of the buffer and PA-β-CD and the applied voltage. The selector concentration was varied between 2.0 and 4.0 mM in 0.2 mM steps. At the optimal CD concentration systematic, 0.30 pH unit changes ranging from 6.20 to 8.30 were carried out. With the optimal CD concentration and pH, the effect of BGE concentration (from 20 to 120 mM by 10 mM changes) on the resolution was investigated. Finally the applied voltage was varied from +15 to +30 kV by 2.5 kV units. The best resolution for the **Tos-preg** (R_S = 2.76) was achieved in the presence of 3.4 mM PA-β-CD at pH 6.80 in 100 mM phosphate buffer with +17.5 kV applied voltage, while for **Dns-preg** R_S = 4.35 was achieved with 3.2 mM PA-β-CD at pH 7.10 in 100 mM phosphate buffer with +17.5 kV voltage (Fig. 4A and B), however for the separation of the dansylated enantiomers, the 50 mM TRIMEB-CD system at pH 2.5 remained the most successful (R_S = 7.74, Fig. 4C). Fig. 5 shows that even 0.1% of the possible R(−) isomer as impurity can be detected with the optimized systems (3.4 mM PA-β-CD at pH 6.8).

The migration order of the enantiomers was determined by spiking the racemic sample with the single S(+) isomer in all experiments. These experiments showed that at pH 9.2 and 7.2 the "S" enantiomers migrate faster. This tendency is not as clear at pH 4.7, since with permethylated CDs the "R" enantiomers migrate faster. At pH 2.5 most CDs (except for TRIMEA- and TRIMEB-CD with **Dns-preg** and P-β-CD with both derivatives) form a more stable complex with the "R" enantiomers, thus this isomer reaches the detector first.

Numerous experiments were attempted to develop a dual CD system for an even better separation of the enantiomers. In these systems an ionized and a neutral CD were mixed at different

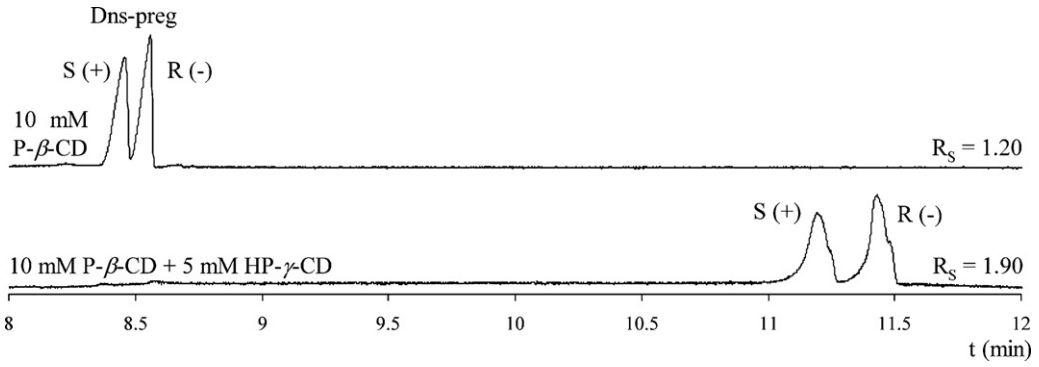

Fig. 6. The electropherograms of Dns-preg with 10 mM P-β-CD and with the dual system of 10 mM P-β-CD and 5 mM HP-γ-CD at pH 2.5.

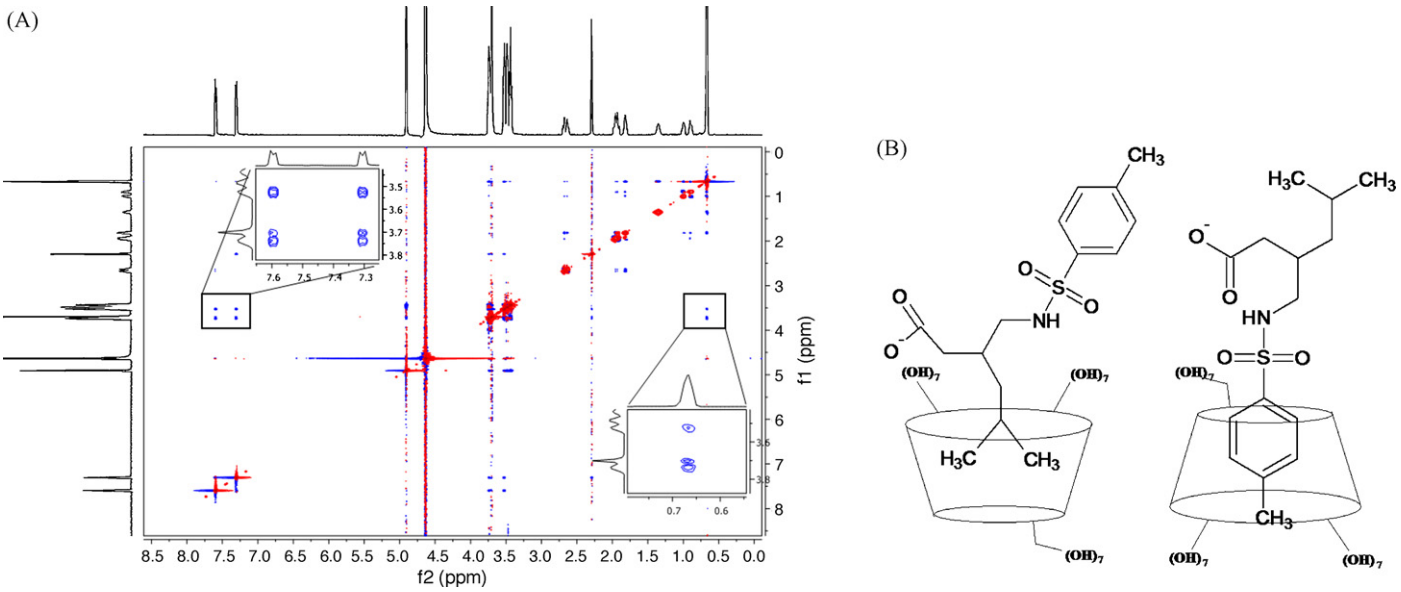

Fig. 8. (A) ROESY spectrum in D_2O with 300 ms mixing time, containing 5 mM Tos-preg and 5 mM β-CD showing the ROE cross-peaks between the aromatic and methyl protons of Tos-preg and 3H, 5H β-CD protons. (B) Two proposed structures for the inclusion complexes of Tos-preg and β-CD based on the ROESY experiments.

concentrations. With adverse enantiomer recognition of the two CDs, better separation could be achieved than with the single components. This effect was unambiguously observed, nevertheless, the previously found highest resolution could not be surpassed. The most promising results were achieved in acidic environment (pH 2.5), where the dual systems improved the separation significantly. P-β-CD alone causes a maximum resolution of $R_s = 1.20$ with **Dns-preg**, while in a dual system of 10 mM P-β-CD and 5 mM HP-γ-CD $R_s = 1.98$ could be achieved. The electropherograms of the above mentioned systems are shown in Fig. 6.

3.2. NMR experiments

3.2.1. The Job's method

The stoichiometry of the inclusion complex was determined with Job plots, using 5 mM guest derivatives and 5 mM β-CD at pH 7.2 in 50 mM phosphate buffer. The stock solutions were mixed at different ratios and chemical shift changes of both the guests and hosts were recorded. The chemical shift displacements ($\Delta\delta$) were then plotted as functions of the molar ratio (see Fig. 7). The resulting Job's plots have a maximum at 0.5 indicating the 1:1 binding stoichiometry for both derivatives.

3.2.2. 2D ROESY NMR

To explore the structure of the inclusion complexes 2D ROESY NMR experiments were carried out. The cross-peaks between CD and pregabalin derivative signals indicate the spatial proximity of these protons as shown in Fig. 8A. These data suggest that two possible structures exist in equilibrium: either the tosyl (or dansyl) moiety gets into the cyclodextrin cavity from the narrower rim (as reported earlier [18]), or the pregabalins' isobutyl moiety enters the CD cavity from the wider rim. The direction of penetration was also confirmed by the different cross-peak intensities obtained for 4-CH_3 of **Tos-preg** and inner CD cavity protons. As suggested by the Job plot experiments these complexes co-exist and are in a rapid exchange on the NMR timescale. Series of ROESY experiments with decreasing mixing time (from 300 to 100 ms by 50 ms steps) show that cross-peaks of aromatic tosyl and inner CD protons are more intense than the cross-peaks of the competing isobutyl methyls'. These data suggest that the aromatic moiety of the guest is the preferred side of inclusion. The possible structures for the complexes of **Tos-preg** and β-CD are depicted in Fig. 8B.

4. Conclusion

Cyclodextrin-hosted diastereomeric complexation and concomitant enantioseparation of pregabalin derivatives and characterization of their inclusion complexes were carried out. UV active tosylated and dansylated derivatives were synthesized for the CE measurements. The binding constants and enantiomeric resolution of the derivatives with 30 different CDs at four pHs were determined. Two of the most promising systems were then optimized in subtle adjustments of pH, buffer and CD concentration and applied voltage. The maximum resolution of the optimized systems is $R_s = 2.76$ for tosylated- and $R_s = 4.35$ for dansylated pregabalin. The highest resolution and therefore the most prominent enantioseparation was achieved at pH 2.5 for Dns-preg enantiomers with TRIMEB-CD (R_s= 7.74). Application of dual CD systems was successful, however achieving no higher resolution than the most suitable single CD systems. The stoichiometry and the structure of the inclusion complexes were determined by NMR experiments. As a result, 1:1 complex is formed with both pregabalin derivatives in two possible orientations: either the aromatic part of the tosyl or dansyl moiety or the isobutyl moiety of the guest fits the CD cavity.

Acknowledgements

This work was supported by the Hungarian Scientific Research Found OTKA K73804. The support of the grant Jedlik Ányos Program 00180/2007 NKFP_07_A3_NATURSEP is gratefully acknowledged.

References

[1] G.K.E. Scriba, Pharmaceutical and biomedical applications of chiral capillary electrophoresis and capillary electrochromatography: an update, Electrophoresis 24 (2003) 2409–2421.
[2] E.L. Izake, Chiral discrimination and enantioselective analysis of drugs: an overview, J. Pharm. Sci. 96 (2007) 1659–1676.
[3] B. Chankvetadze, Enantioseparations by using capillary electrophoretic techniques. The story of 20 and a few more years, J. Chromatogr. A 1168 (2007) 45–70.
[4] Z. Juvancz, R.B. Kendrovics, R. Iványi, L. Szente, The role of cyclodextrins in chiral capillary electrophoresis, Electrophoresis 29 (2008) 1701–1712.

[5] J. Szejtli, Introduction and general overview of cyclodextrin chemistry, Chem. Rev. 98 (1998) 1743–1753.
[6] J.S. Bryans, D.J. Wustrow, 3-Substituted GABA analogs with central nervous system activity: a review, Med. Res. Rev. 19 (1999) 149–177.
[7] R. Freynhagen, K. Strojek, T. Griesing, E. Whalen, M. Balkenohl, Efficacy of pregabalin in neuropathic pain evaluated in a 12-week, randomised, double-blind, multicentre, placebo-controlled trial of flexible- and fixed-dose regimens, Pain 115 (2005) 254–263.
[8] R.H. Dworkin, A.E. Corbin, J.P. Young Jr., U. Sharma, L. LaMoreaux, H. Bockbrader, E.A. Garofalo, R.M. Poole, Pregabalin for the treatment of postherpetic neuralgia: a randomized, placebo-controlled trial, Neurology 60 (2003) 1274–1283.
[9] L.J. Crofford, M.C. Rowbotham, P.J. Mease, I.J. Russell, R.H. Dworkin, A.E. Corbin, J.P. Young Jr., L.K. LaMoreaux, S.A. Martin, U. Sharma, Pregabalin for the treatment of fibromyalgia syndrome. Results of a randomized, double-blind, placebo-controlled trial, Arth. Rheum 52 (2005) 1264–1273.
[10] R.B. Silverman, From basic science to blockbuster drug: the discovery of Lyrica, Angew. Chem. Int. Ed. 47 (2008) 3500–3504.
[11] U. Mandal, A.K. Sarkar, K.V. Gowda, S. Agarwal, A. Bose, U. Bhaumik, D. Ghosh, T.K. Pal, Determination of pregabalin in human plasma using LC–MS–MS, Chromatographia 67 (2008) 237–243.
[12] D. Berry, C. Millington, Analysis of pregabalin at therapeutic concentrations in human plasma/serum by reversed-phase HPLC, Ther. Drug Monit. 27 (2005) 451–456.
[13] T.A.C. Vermeij, P.M. Edelbroek, Simultaneous high-performance liquid chromatographic analysis of pregabalin, gabapentin and vigabatrin in human serum by precolumn derivatization with o-phtaldialdehyde and fluorescence detection, J. Chromatogr. B 810 (2004) 297–303.
[14] S. Lin, P.L. Brower, D.D. Winkle, Chiral HPLC separations for process development of S-(+)-isobutyl GABA, a potential anti-epileptic agent, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2699–2708.
[15] Y. Zhang, C. Holliman, D. Tang, D. Fast, S. Michael, Development and validation of a direct enantiomeric separation of pregabalin to support isolated perfused rat kidney studies, J. Chromatogr. B 875 (2008) 148–153.
[16] A.S. Jadhav, D.B. Pathare, M.S. Shingare, Validated enantioselective LC method, with precolumn derivatization with Marfey's reagent, for analysis of the antiepileptic drug pregabalin in bulk drug samples, Chromatographia 65 (2007) 253–256.
[17] X. Chen, D. Zhang, J. Deng, X. Fu, Determination of optical impurity of pregabalin by HPLC with pre-column chiral derivatization, J. Chromatogr. Sci. 46 (2008) 42–44.
[18] M. Yoshinaga, M. Tanaka, Use of selectively methylated β-cyclodextrin derivatives in chiral separation of dansylamino acids by capillary zone electrophoresis, J. Chromatogr. A 679 (1994) 359–365.
[19] X. Deng, N.S. Mani, A facile, environmentally benign sulfonamide synthesis in water, Green Chem. 8 (2006) 835–838.
[20] M.S. Hoekstra, D.M. Sobieray, M.A. Schwindt, T.A. Mulhern, T.M. Grote, B.K. Huckabee, V.S. Hendrickson, L.C. Franklin, E.J. Granger, G.L. Karrick, Chemical Development of CI-1008, an enantiomerically pure anticonvulsant, Org. Process Res. Dev. 1 (1997) 26–38.
[21] R.A. Wallingford, A.G. Ewing, Capillary electrophoresis, Adv. Chromatogr. 29 (1989) 1–76.
[22] K.L. Rundlett, D.W. Armstrong, Examination of the origin, variation, and proper use of expressions for the estimation of association constants by capillary electrophoresis, J. Chromatogr. A 721 (1996) 173–186.
[23] M. Kyvala, I. Lukes, OPIUM computer program, Chemometrics'95 Pardubice (1995) Book of abstracts 63. http://www.natur.cuni.cz/~kyvala/opium.html.
[24] S.A.C. Wren, R.C. Rowe, Theoretical aspects of chiral separation in capillary electrophoresis. I. Initial evaluation of a model, J. Chromatogr. 603 (1992) 235–241.