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Fast chiral separations using sulfated β-cyclodextrin and short-end injection in capillary electrophoresis

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Abstract

The general applicability of sulfated β-cyclodextrin as chiral selector and short-end injection in capillary electrophoresis (CE) as a powerful screening tool for fast and efficient chiral separation of Ormeloxifene enantiomers and racemic Ormeloxifene analogues is demonstrated. Using the short-end injection procedure, all of the 16 racemic compounds studied were successfully separated with high efficiencies and with analysis times of less than 1.2 min. Furthermore, long-end injections of eight analogues named C1–C8 afforded separations with extremely high efficiencies. A statistical evaluation of the resolution values obtained in short-end and long-end injections of compounds C1–C8 showed that the sensitivity of the CE method towards structural changes in the studied molecules is intact when the chiral analysis is performed with short-end injection compared to conventional long-end injection. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Injection methods; Short-end injection; Ormeloxifene; Cyclodextrins; Sulfated cyclodextrin

1. Introduction

(−)-Ormeloxifene is a non-steroid partial estrogen receptor agonist that was selected as a drug candidate for the prevention and treatment of postmenopausal osteoporosis. During the development of this drug, a variety of structurally related compounds such as racemic metabolites, degradation products and synthesis impurities were produced, and a screening tool for separation of optical isomers was needed. On the basis of our previous work with separation of Ormeloxifene enantiomers using capillary electrophoresis (CE) [1], we discovered that one method was ideal for rapid screening of compounds which are structurally related to Ormeloxifene.

This paper describes the general applicability of sulfated β-cyclodextrin (sulfated β-CD) as chiral selector and short-end injection in CE for fast and efficient chiral separation of Ormeloxifene enantiomers (C1) and racemic Ormeloxifene analogues (C2–C16) (Fig. 1 and Table 1).

Speed has become a crucial factor in research and development of new pharmaceuticals and fast analytical methods for chiral separations are necessary for evaluation, characterization and control of chiral drug candidates. CE represents a powerful analytical tool for the separation of chiral compounds due to the high efficiencies and short analysis times that can be achieved [2–7]. In addition, the availability of various types of chiral selectors and the need for minimal amounts of sample and electrolyte in CE, allow rapid and inexpensive method development

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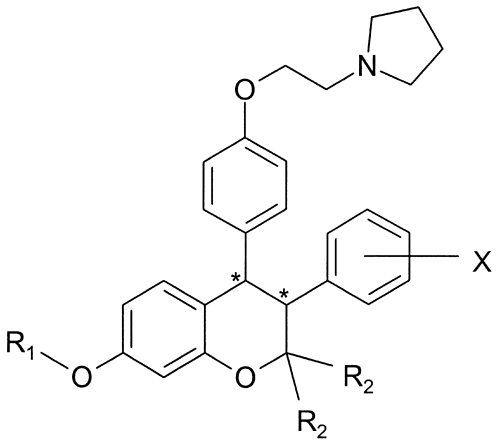

Fig. 1. Basic structural unit of racemic compounds analyzed and listed in Table 1.

compared to high-performance liquid chromatography (HPLC) [8,9]. In CE, injection is usually performed at the end of the capillary longest from the detector, and separation of enantiomers in the minutes range is possible [10,11]. However, the analysis time can be reduced significantly by using a short-end injection procedure where the capillary length used for separation is decreased by injecting the sample from the end of the capillary nearest to the detector. The application of the short-end for separation was first introduced by Mazzeo and Krull [12] for analysis of proteins by capillary isoelectric focusing (CIEF). Recently, Altria et al. demonstrated the benefits of using short-end injection in CE for rapid analysis of pharmaceuticals, and enantiomer separations within 1–2 min were achieved [13]. With the short-end injection technique in combination with sulfated β-CD as chiral selector we have shown that separation of Ormeloxifene enantiomers (C1) can be obtained in the seconds range [1]. Other ways to reduce analysis time in CE are using short total capillary lengths [14–16] and/or high voltages [15]. However, with these approaches high currents may be a problem, and typical commercial CE instruments limits the minimum total length of capillary to around 17–65 cm [17].

Sulfated β-CD has previously been used with success as isomer selector in various chiral CE separations [18–23]. Especially when analyzing basic racemic drugs such as Ormeloxifene and its analogues, negatively charged sulfated β-CD enables better enantiomeric resolution of analytes because the chiral selector and free analyte migrate in opposite directions [18,19].

In this paper we demonstrate that the combination of sulfated β-CD as chiral selector and short-end injection in CE is a powerful screening tool for fast

Table 1
Chemical structures of racemic Ormeloxifene (C1) and racemic Ormeloxifene analogues (C2–C16) (the basic structural unit is shown in Fig. 1)

Compound	R_1	R_2	X	Absolute configuration of racemate
C1	Me	Me	–	3,4-(*R*,*R*) and (*S*,*S*) (*trans*)
C2	Me	Me	–	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C3	H	Me	–	3,4-(*R*,*R*) and (*S*,*S*) (*trans*)
C4	H	Me	–	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C5	Me	H	–	3,4-(*R*,*R*) and (*S*,*S*) (*trans*)
C6	Me	H	–	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C7	H	H	–	3,4-(*R*,*R*) and (*S*,*S*) (*trans*)
C8	H	H	–	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C9	H	Me	*m*-Me	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C10	H	Me	*p*-Me	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C11	H	Me	*m*-CF_3	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C12	H	Me	*p*-CF_3	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C13	H	Me	*m*-OMe	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C14	H	Me	*p*-OMe	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C15	H	Me	*p*-F	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)
C16	H	Me	per fluoro	3,4-(*R*,*S*) and (*S*,*R*) (*cis*)

separation of optical isomers which are structurally related to Ormeloxifene (Fig. 1 and Table 1).

2. Experimental

2.1. Instrumentation

A HP^{3D}CE capillary electrophoresis instrument (Hewlett-Packard, Waldbronn, Germany) was used in all experiments. The instrument was equipped with an autosampler, a capillary cartridge, a high-voltage power supply, a diode array detector, electrodes and a hydrostatic injection system allowing injections into both ends of the capillary. CE separations were carried out using untreated fused-silica capillaries of 58.5 cm×50 μm I.D. from Composite Metal Services (Hallow, UK). Every new capillary was preconditioned before use by rinsing for 30 min with 1 *M* sodium hydroxide. The electrophoretic data system was the HP Chemstation software (Hewlett-Packard) operated on a HP Vectra pentium computer (Hewlett-Packard). Data were collected at a rate of 10 data points per s.

2.2. Materials

Sulfated β-CD (typical substitution 7–11 mol/mol β-CD) was purchased from Aldrich (Milwaukee, WI, USA). During this study the same lot of sulfated β-CD was used, and it should be noticed that the cyclodextrin product is not pure and can be variable. Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and orthophosphoric acid (H_3PO_4) of analytical quality and HPLC grade methanol were obtained from Merck (Darmstadt, Germany). Water was obtained from a Millipore Milli-Q deionizer system (Millipore, Bedford, MA, USA).

Racemic Ormeloxifene (C1) and racemic Ormeloxifene analogues (C2–C16) were prepared by the Chemical Development Department at Novo Nordisk A/S (Copenhagen, Denmark). The absolute configuration of (−)-Ormeloxifene has been determined to be 3,4-(*R*,*R*) by X-ray crystallography.

2.3. Analysis procedures

A 10 m*M* sodium dihydrogenphosphate solution (pH 3.0) was used as buffer. For CE experiments, the electrolyte was prepared by dissolving 2.0% (w/v) sulfated β-CD in buffer, adjusting to pH 3.0 with 0.1 *M* orthophosphoric acid and filtering through a 0.45-μm polypropylene filter (Whatman, Ann Arbor, MI, USA).

Standard stock samples were approximately 0.5 mg/ml racemic compound in methanol, and standard samples for CE analysis were prepared by mixing 100 μl standard stock sample and 1000 μl buffer to yield a final concentration of 0.023 mg/ml of each enantiomer.

Every day before use, the capillary was conditioned by treatment with water for 5 min, 0.1 *M* sodium hydroxide for 5 min and water for 5 min. Between runs the capillary was equilibrated with electrolyte for 1 min.

2.3.1. Short-end injection

In short-end injection, the sample is injected from the end of the capillary nearest the detector. With the used equipment this was performed applying negative pressure. For successful separation when using the short-end of the capillary, the CE instrument was operated in the positive polarity mode meaning that the detection-end electrode was the cathode.

2.3.2. Long-end injection

Conventional injection or long-end injection where injection of sample is done at the end of the capillary furthest away from the detector was performed using positive pressure. Electrophoresis was done with negative polarity voltage.

When the short-end of the capillary was employed for separation the capillary was not thermostatted since the short-end was outside the capillary cartridge. The experimental CE parameters for the injection types are listed in Table 2.

2.4. Calculations

For each chiral separation, efficiency in terms of theoretical plates, N, and resolution, R_s, was calculated according to Ph. Eur. Third Ed. Section 2.2.29. Peak areas were corrected by dividing the obtained peak area by the corresponding migration time.

The effects on the resolution values, R_s, obtained for the chiral separations of Ormeloxifene enantio-

Table 2
CE parameters used for short-end and long-end injections

Condition	Short-end injection	Long-end injection
Total capillary length (cm)	58.5	58.5
Internal capillary diameter (μm)	50	50
Effective capillary length (cm)	8.5	50.0
Hydrostatic sample injection	−15 mbar, 2.0 s	+50 mbar, 3.0 s
Polarity (kV)	+25	−15
Current (μA)	+77	−32
Detection wavelength (nm)	205	205
Temperature (°C)	Ambient	30

mers (C1) and racemic analogues (C2–C8) were evaluated statistically by analysis of variance (ANOVA). R_s values are not normal distributed because R_s values can never be less than zero, and therefore the log-transformed R_s values were used for the statistical analyses. All statistical calculations were performed using Statgraphics Plus for Windows 2.1 (Manugistics, Rockville, MD, USA).

3. Results and discussion

3.1. Principle of separation

At pH 3.0 sulfated β-CD is dissociated and polynegatively charged while the racemates (C1–C16) are positively charged due to protonation of the pyrrolidine moiety in the basic structural unit (Fig. 1). Thus, with the CE conditions described in the experimental section sulfated β-CD migrates toward the detector (anode) whereas free racemic analytes migrate in the opposite direction (cathode). Since the electroosmotic flow is negligible at pH 3.0, a countercurrent flow of chiral selector and free racemic analytes exists in the capillary. At the same time the chiral selector forms complexes with analytes, and these complexes migrate in the direction of the detector (anode) because the effective electrophoretic mobilities are negative. Separation of the enantiomers depends on the difference in the binding constants of the equilibrium between free enantiomer and complex. If a difference between the binding constants exists then countercurrent flow enables better enantiomer separation of both weakly and strongly interacting racemates, since the mobility difference between free enantiomer and complex is increased.

Table 3
Results obtained by short-end injection of the racemates (N_1 and t_1 are theoretical plate number and migration time, respectively, of the first detected enantiomer peak)

Compound	t_1 (s)	t_2 (s)	N_1	N_2	R_s
C1	43.2	63.6	44 000	12 000	13.0
C2	45.0	58.8	33 000	17 000	9.9
C3	41.4	72.6	49 000	32 000	26.5
C4	43.8	65.4	41 000	21 000	16.5
C5	41.4	46.8	36 000	20 000	5.0
C6	42.0	46.8	33 000	12 000	3.4
C7	41.4	47.4	39 000	17 000	5.4
C8	42.0	46.2	38 000	19 000	3.7
C9	67.2	70.8	45 000	45 000	1.5
C10	66.6	70.8	47 000	47 000	1.8
C11	66.0	69.6	44 000	44 000	1.5
C12	66.0	70.2	60 000	60 000	2.4
C13	69.0	72.0	50 000	50 000	1.5
C14	65.4	68.4	50 000	50 000	2.0
C15	65.4	67.8	48 000	48 000	2.1
C16	67.2	69.6	40 000	40 000	1.3

The interaction between polynegatively charged sulfated β-CD and cationic racemates (C1–C16) is probably due to hydrophobic driven inclusion complexation as well as electrostatic interactions that may stabilize the complexes [18,19]. In addition to the enhanced enantioresolving ability of countercurrent flow, the opposite direction of mobility of sulfated β-CD and free racemates (C1–C16) results in sample concentration also known as stacking [24], which may explain the high efficiencies and enantiomeric resolutions that are obtained in this study.

3.2. Short-end injection

In Table 3 migration times (t_1 and t_2) and ef-

ficiency data (N_1, N_2 and R_s) obtained for the chiral separations using short-end injection are shown. Compounds C1–C8 were successfully resolved with notable high resolutions (R_s) while separation of compounds C9–C15 were satisfactory. Compound C16 was the only racemate which was only partly resolved. However, theoretical plate numbers (N_1 and N_2) for all enantiomer peaks were in the range of 12 000 to 60 000 and analysis times were very fast, since all racemates were separated in less than 75 s. As can be seen from Table 3, the best separation was achieved for compound C3 with a resolution of 26.5 and analysis time of 73 s. The fastest analysis time was that of compound C8 where the enantiomers were resolved within 46 s. As we have shown previously, analysis times can be reduced by applying greater electric field strength across the capillary by either decreasing the total length of capillary and/or increasing the applied voltage. Using the instrumental limits (33.0 cm total capillary length and +30 kV) it is possible to separate compound C1 in approximately 40 s [1]. However, for the purpose of screening optical isomer analogues of Ormeloxifene the instrumental set-up resulting in analysis times of less than 75 s was more than satisfactory. Representative electropherograms of short-end separations are shown for racemates C2 (Fig. 2a) and C3 (Fig. 2b). Racemate C2 is the corresponding *cis* configuration of racemic Ormeloxifene C1 (*trans* configuration). Separation of all four stereoisomers in the same analysis is possible as indicated by the migration times in Table 3.

Generally, the method displays high enantioselectivity for racemates C1–C8, but dramatically lower enantioselectivity for racemates C9–C16. Thus, the combination of substituents (X) on the phenyl ring at position 3 (Fig. 1) and R_1=H/R_2=Me diminishes resolution as indication of weak interaction between these enantiomers and cyclodextrin. Table 3 also shows that compounds with *para* substitutions (C9, C11 and C13) are better separated than the corresponding *meta* substituted compounds (C10, C12 and C14).

From Fig. 2 it is noted that the last detected enantiomer is a lower and broader peak than the first detected enantiomer peak. Furthermore, integration results of separations of compounds C1–C8 consistently show that the corrected enantiomer peak

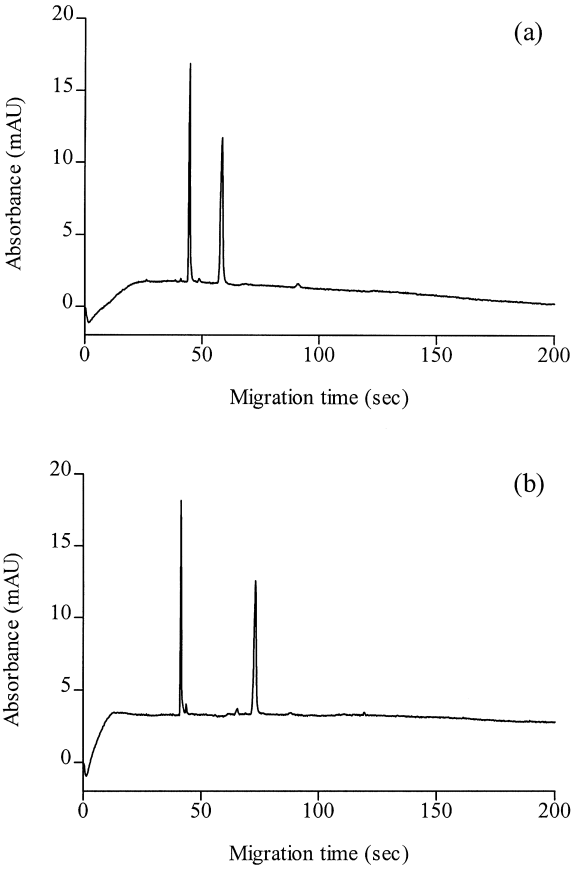

Fig. 2. Short-end injection electropherograms of (a) compound C2 (*cis* configuration) and (b) compound C3 (*trans* configuration). Electrolyte: 2.0% (w/v) sulfated β-CD in 10 m*M* NaH_2PO_4, pH 3.0 buffer. Capillary dimensions: 58.5 cm×50 μm. Effective capillary length: 8.5 cm. Sample injection: 2.0 s at −15 mbar. Voltage: +25 kV. Current: +77 μA. Detection: UV at 205 nm. Temperature: ambient.

area of the first detected enantiomer peak exceeds that of the second detected enantiomer peak. This observation of unequal enantiomer peak areas for racemates C1–C8 may be explained by large differences in the binding constants of the respective enantiomer–cyclodextrin equilibria. A much greater affinity of sulfated β-CD towards one of the enantiomers results in the first peak where the concentration of sulfated β-CD is larger than in the second peak. If we assume that sulfated β-CD absorbs at the detection wavelength (205 nm), the first peak area will be larger than the second peak area. The problem with unequal enantiomer peak areas can be solved by

detecting at a higher wavelength where the UV absorbtion of sulfated β-CD is insignificant. For quantitative measurements the response factor should be determined to correct the results. Also, the unequal peak shapes may partly be due to increased dispersion because the last migrating peak is exposed to diffusion processes for a longer time in the capillary. Regarding compounds C9–C16 unequal peak areas and shapes were negligible as indication of small differences in binding constants. In accordance with these observations the calculated efficiencies in terms of theoretical plate numbers (Table 3) show that $N_2 < N_1$ for the highly resolved racemates (C1–C8), whereas for racemates C9–C16, $N_2 = N_1$ for each separation.

3.3. Long-end injection

To evaluate the sulfated β-CD based short-end injection method, compounds C1–C8 were analyzed from the long-end of the capillary. The long-end injection results are given in Table 4. Extremely high efficiencies were observed, since theoretical plate numbers as high as 680 000 and enantiomeric resolutions of more than 100 were achieved. However, analysis times increased with over one order of magnitude and separations were completed in the minutes range instead of the seconds range. Nevertheless, the results showed the same trend as those obtained with the short-end injection experiments of racemates C1–C8. Example of a long-end injection electropherogram is shown for compound C2 in Fig. 3.

Unequal peak shapes and corrected peak areas of the resolved enantiomers were more pronounced for the long-end injections compared to the short-end injections (Figs. 2a and 3).

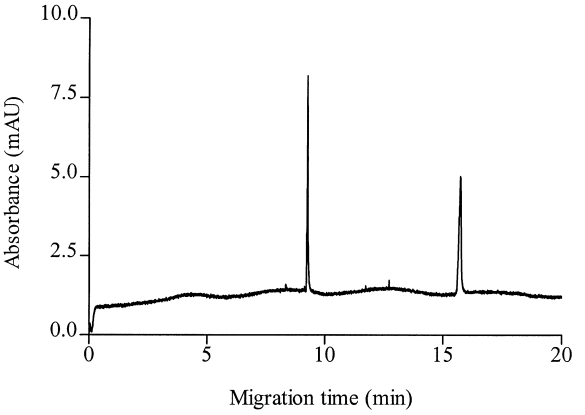

Fig. 3. Long-end injection electropherogram of compound C2. Electrolyte: 2.0% (w/v) sulfated β-CD in 10 m*M* NaH_2PO_4, pH 3.0 buffer. Capillary dimensions: 58.5 cm×50 μm. Effective capillary length: 50.0 cm. Sample injection: 3.0 s at +50 mbar. Voltage: −15 kV. Current: −32 μA. Detection: UV at 205 nm. Temperature: 30°C.

Table 4
Results obtained by long-end injection of racemates C1–C8 (N_1 and t_1 are theoretical plate number and migration time, respectively, of the first detected enantiomer peak)

Compound	t_1 (min)	t_2 (min)	N_1	N_2	R_s
C1	8.02	18.45	687 000	198 000	102.0
C2	9.29	15.74	374 000	140 000	56.2
C3	7.59	21.82	659 000	173 000	113.0
C4	8.30	17.63	496 000	148 000	80.7
C5	7.55	9.39	510 000	378 000	35.4
C6	7.66	9.25	296 000	389 000	27.4
C7	7.45	9.53	633 000	320 000	39.5
C8	7.58	8.89	346 000	208 000	20.2

3.4. Short-end injection versus long-end injection

The short-end and long-end injection results of racemates C1–C8 represented an excellent opportunity to study the difference between long-end and short-end injections and the sensitivity of the CE method to variations in the molecular structure. Thus, the R_s values of compounds C1–C8 in Tables 3 and 4 formed the basis for a statistical evaluation by ANOVA. Since all structural combinations of racemates (C1–C8) were analyzed using both long-end and short-end injections, the total of 16 runs gave the structure of a complete 2^4 factorial design as outlined in Table 5.

The log-transformed R_s values were used for the statistical testing in order to make the R_s data

Table 5
Experimental design used for the statistical evaluation

Factor	Level 1	Level 2
(A) Substitution on R_1	H	Me
(B) Substitution on R_2	H	Me
(C) *cis* or *trans*	*cis*	*trans*
(D) Injection	Short-end	Long-end

approximately normal distributed and variance homogeneous. Main results of the ANOVA are illustrated in Fig. 4.

On the basis of the four factors described in Table 5, interpretation of the statistical analysis focuses on the sensitivity of the CE method towards structural changes in the molecule (factors A–C) and the difference between long-end and short-end injections (factor D).

Fig. 4 clearly shows that factors B and C, have statistical significant influence on the resolution, because 95% confidence intervals are not overlapping. Apparently, when the substitution on R_2=Me and the molecular structure around the chiral centers are the *trans* configuration, highest resolutions are achieved. Regarding factor A, the effect on the resolution is statistical significant for the short-end injections but not for the long-end injections. The ANOVA shows that the studied molecular changes have significant independent impact on the chiral resolution. The fact that relatively remote molecular methylations (factors A and B) have such significant effect on the resolution, indicates the existence of a number of different analyte–cyclodextrin complexes

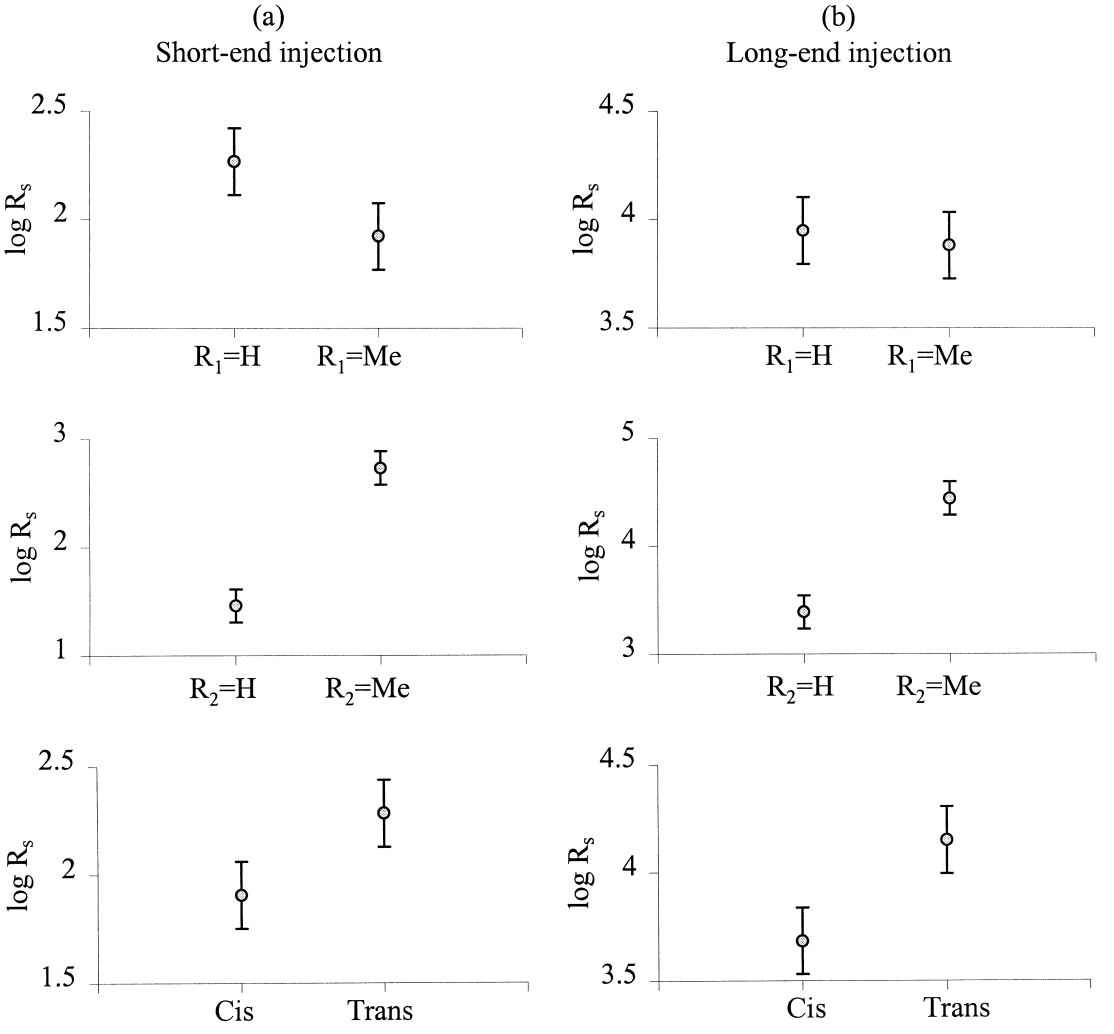

Fig. 4. Means and 95% confidence intervals of the resolution values (log-transformed R_s values) for long-end and short-end injections of compounds C1–C8.

as described by the model proposed by Sänger-van de Griend et al. [25].

Comparison of short-end injection (Fig. 4a) and long-end injection (Fig. 4b) shows that R_s values are generally greater for long-end injections (factor D). However, more importantly, it is observed that regardless of the injection type, the trends in the results for factors A–C are identical which means that, the sensitivity of the CE method towards structural changes in the studied molecules is intact when the chiral analysis is performed with short-end injection compared to long-end injection. This justifies the use of short-end injection with the sulfated β-CD based CE method, since the same effects are observed when applying both the short-end and long-end of the capillary.

4. Conclusions

All of the 16 racemic compounds studied have been successfully separated with high efficiencies and with analysis times of less than 1.2 min. Moreover, a statistical evaluation of the resolution values obtained in short-end and long-end injections of compounds C1–C8 show that the sensitivity of the CE method towards structural changes in the studied molecules is intact when the chiral analysis is performed with short-end injection compared to conventional long-end injection.

Therefore, the combination of sulfated β-CD as chiral selector and short-end injection in CE is an ideal screening tool for fast separation of optical isomers analogues of Ormeloxifene. The screening process will indicate if a compound can be resolved, and the separation of an interesting compound may be optimized applying the long-end of the capillary if extra resolution is needed. In this way, using CE and short-end injection, development and validation of chiral purity methods have become considerably faster.

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