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Improved quantification limits in chiral capillary electrochromatography by peak compression effects

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Abstract

The peak compression effect has been applied to improve quantification limits in chiral capillary electrochromatography (CEC). A stationary phase based on the chiral selector vancomycin (Chirobiotic V) was used for separations of the enantiomers of mianserin. By adding solvents with a low dielectric constant, e.g. 2-propanol or tetrahydrofuran, to the sample solution, peak compression could be induced. The plate numbers for the minor enantiomer increased from approximately 100,000 to 1.4–1.6 million plates/m, when the composition of the mobile phase was adjusted so that the analyte eluted within either one of two system zones originating from the sample solution. A 10-fold improvement in the quantification limit for the minor enantiomer was obtained compared to elution under non-focused conditions.

Keywords: Electrochromatography; Enantiomer separation; Peak compression; Chiral stationary phases, electrochromatography; System zones; Mianserin; Vancomycin

1. Introduction

The peak compression effect utilizing a strong cation exchanger (SCX) in capillary electrochromatography (CEC), with apparent efficiencies of up to 8 million plates/m, was first reported in 1995 by Smith and Evans [1]. This paper raised hopes that very efficient separations might be obtained by using CEC. However, the effect was found to be difficult to explain and to control and suffered from lack of reproducibility [2–4]. Peaks with high apparent efficiencies have also been reported for CEC under reversed phase conditions [5]. Non-equilibrium pulses were caused by differences between the solvent compositions in the sample and the mobile phase. When the migration time of the partially anionic/neutral analytes closely matched the elution time of sample induced discontinuities in the mobile phase, the peak efficiency could reach 2.5 million plates/m. It has also been shown that peak compression effects can be induced in liquid chromatography (LC) due to the composition of the sample solvent [6]. In this study, alkane sulfonate ions, added to the sample, formed ion-pairs with dimethyloctylamine (DMOA) in the mobile phase, creating an ion-pair zone and a DMOA-depleted zone. When an analyte eluted close to the DMOA-depleted zone, the efficiency was much higher than for the rest of the analytes.

Our research group has reported results in a recent publication [7], where reproducible peak compression effects were obtained in CEC on a SCX column by utilizing differences in the composition of the sample solvent and the mobile phase for the analysis of a number of basic drug substances. A mechanism behind the peak compression phenomena was proposed which we called “continuous stacking”. The continuous stacking occurred when non-equilibrium conditions were introduced, involving lower retention and higher electrophoretic velocity within the sample zone as compared to the mobile phase. In the SCX–CEC system, this occurred when the analyte eluted rather close to the electroosmotic flow (EOF) marker, the sample solution contained a higher percentage of acetonitrile than the mobile phase and a sufficiently large injection volume had been used. This introduced a sample zone where the electric field strength was locally higher and therefore the positively charged analytes were less retained, which resulted in a higher elution rate through this zone than in the rest of the system. When the analyte exits this zone and enters the mobile phase, it interacts more strongly

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with the stationary phase and slows down, and the sample zone can catch up with the analyte and the process starts all over again. Hence, a continuous stacking occurs at the boundary between the sample zone and the mobile phase resulting in very narrow peaks with high apparent efficiencies.

Unfortunately, this peak compression effect proved to be of limited use for improving separation as some of the peaks co-eluted under peak compression conditions but not under normal conditions. However, the continuous stacking effect could be utilized to improve quantification limits and the principle of the mechanism ought to be applicable on stationary phases other than SCX. A particularly interesting area to make use of this peak compression effect would be in chiral analysis. As the enantiomers of drug substances may have different pharmacological effects and/or activities in the human body, it is often a requirement to synthesize enantiomerically pure drug substances and be able to determine the amount of the undesired enantiomer present down to very low levels.

Chiral analysis is mainly performed by separation of enantiomers using chromatographic or electromigration techniques [8,9]. A fairly new option is chiral CEC, which can be performed in a number of ways as described in recent reviews [10–12]. Vancomycin, a macrocyclic antibiotic, has been used as a chiral selector in LC since the mid-1990s and has demonstrated a broad selectivity in reversed phase, normal phase and polar organic mode [13]. Vancomycin has also been used in chiral CEC [14–19] with, in some cases, very high resolution of the enantiomers. This was the main reason why this type of stationary phase was selected for the study of the peak compression effect as a method for improving quantification limits.

2. Experimental

2.1. Chemicals

Buffer stock solutions with a pH of 4.8 were made from acetic acid, triethylamine or sodium hydroxide, and water (HPLC grade). The ionic strength was calculated in all the buffer stock solutions. Mobile phases were made from buffer stock solution, water and acetonitrile (gradient grade) and degassed by purging with helium. The pH in the buffer was measured before addition of acetonitrile. Racemic mianserin hydrochloride was purchased from Sigma (St. Louis, MO, USA) and stock solutions (0.5 mg/ml) were, depending on the experiments, made in acetonitrile or 2-propanol.

Preparative LC was used to isolate the enantiomers. Mianserin hydrochloride (5 mg/ml) was injected (100 μl) on a Chiralpak AD column (4.6 mm × 250 mm; Daicel Chemical Industries, Tokyo, Japan). The mobile phase, which consisted of methanol, ethanol and diethylamine (50:50:0.1), had a flow rate of 1 ml/min. The two fractions containing the separated enantiomers of mianserin were evaporated under nitrogen flow and the residues were dissolved in 2-propanol and stored in a refrigerator.

2.2. Instrumentation

The experiments were performed with fused-silica capillaries obtained from Polymicro Technologies (Phoenix, Arizona, USA). The dimensions of the capillaries were 100 μm i.d., 360 μm o.d., with an effective packed length of 25 cm. The length after the detector was 8.5 cm. The capillaries were mounted in an Agilent CE system (Agilent Technologies, Waldbronn, Germany). To suppress bubble formation, both ends of the column were pressurized with nitrogen at 1.0 MPa. UV detection was mainly done at 214 nm, and diode array spectra between 190 and 300 nm were collected to ensure peak identity. An HPLC pump (1100 series, Agilent Technologies) with a split creating a back-pressure of 10.0 MPa at a flow of 1 ml/min was used to purge the column. The samples were electrokinetically injected towards the cathode and a 5 kV × 10 s plug of mobile phase was injected behind the sample. The column was thermostated at 15 °C. The voltage was 25 kV (ramp 0.3 min) resulting in currents of 5–8 μA depending on the mobile phase.

2.3. Column preparation

The packing material Chirobiotic V (5 μm) was purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA) and columns were packed according to a method previously described in detail [20,21].

2.4. Evaluation of efficiency

The values for efficiency (N) were calculated by Agilent ChemStation using the following equation:

$$N = 5.54 \times \frac{t_r^2}{w_{0.5}^2} \quad (1)$$

where t_r is the retention time and $w_{0.5}$ the width at half height.

3. Results and discussion

As mentioned earlier, one criterion for peak compression to occur in the SCX–CEC system was that the analyte should elute rather closely to the EOF marker. The retention of the analyte could be controlled by varying the composition of the mobile phase (i.e. pH, ionic strength and amount of organic modifier). It was also necessary for the analyte to have a higher velocity in the sample zone compared to the mobile phase. This could be achieved by employing a higher content of acetonitrile in the sample solution than in the mobile phase. This causes lower retention and a locally increased electric field in the sample zone. Moreover, comparatively

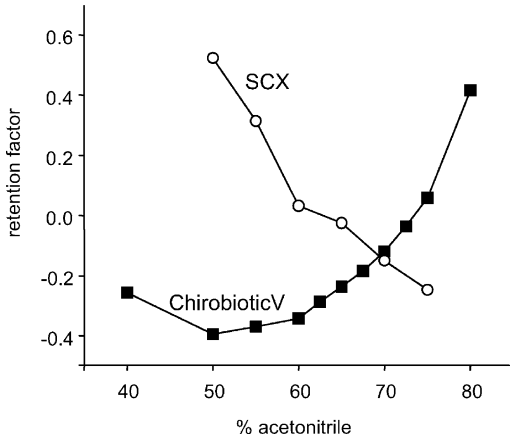

Fig. 1. The effect of acetonitrile content in the mobile phase on retention of ropivacaine on the SCX system and mianserin on the Chirobiotic V system. The retention factor was calculated according to $k = (t_{mig} - t_{eof})/t_{eof}$.

large injection volumes were necessary to induce peak compression.

Mianserin was used as model compound in this study since its enantiomers have previously been very well resolved on a vancomycin-based column in reversed phase mode [18]. Under the assumption that the same conditions were needed to induce peak compression in a vancomycin-based system as in a SCX system, the process of finding the proper conditions was commenced. First, the composition of the mobile phase was altered in such a way that the enantiomers of mianserin eluted rather closely behind the EOF marker (acetone). However, contrary to our previous experience, the increase of the acetonitrile content in the sample solution combined with an increase of the injection volume did not result in peak compression. To find out why this had happened, the effect of acetonitrile content on the retention and EOF was studied (while keeping the buffer concentration in the mobile phase constant). A quite different result was found on the vancomycin-based column compared to the SCX column. On the SCX column, the retention factor (defined as $(t_{mig} - t_{eof})/t_{eof}$), as well as the EOF, decreased with increased content of acetonitrile (Fig. 1). On the Chirobiotic V column, a reduction in retention factor was observed while the EOF was virtually unchanged when increasing the acetonitrile content up to 50%, while any further increase in the percentage of the organic modifier resulted in increased retention factor and EOF (Fig. 1). This implied that a higher acetonitrile content of the sample solution, than the 75% used in the mobile phase, would not result in lower retention in the sample zone. Further, using the acetonitrile concentration in the sample zone that would create the lowest retention (50%) also did not produce peak compression (Fig. 2A). This was probably because the conductivity/electric field strength in the sample zone did not differ enough compared to that in the mobile phase and therefore there was not a substantially higher electrophoretic velocity in the sample zone. The small difference

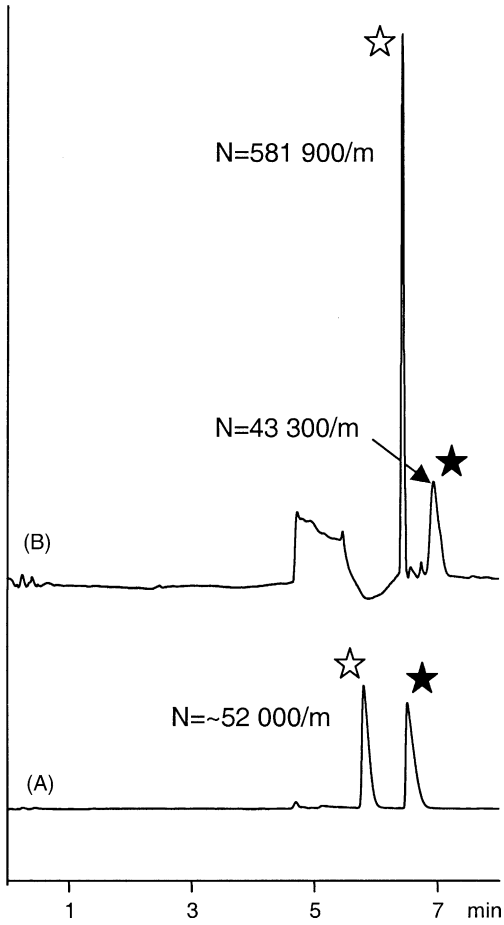

Fig. 2. Peak compression was accomplished when the sample contained 2-propanol. Sample A: 59 μM mianserin dissolved in 50% acetonitrile. Sample B: 59 μM mianserin dissolved in 28% 2-propanol and 38% acetonitrile. Mobile phase: TEAA buffer, pH 4.8, 75% acetonitrile, ionic strength 9.4 mM. Electrokinetic injection 10 kV × 10 s; voltage 25 kV; current 7.7 μA.

in conductivity was also indicated by the current being virtually unchanged even when large injection volumes were used.

Differences in conductivity in the sample zone relative to the mobile phase can be achieved by altering the ionic strength and/or by adding solvents with different dielectric constants. It was found that addition of 2-propanol, which has half the dielectric constant of acetonitrile, to the sample solution produced the desired peak compression effect (Fig. 2B). The efficiency of the sharpened peak was above 580,000 plates/m, under these non-optimized conditions. This is not as extreme as was seen on the SCX columns, but this is still much higher than normally obtained on the Chirobiotic V column. To understand if this observed peak compression was caused by the low dielectric constant or the high viscosity of 2-propanol as compared to acetonitrile, a new experiment was carried out. Tetrahydrofuran, which has an even lower viscosity and dielectric constant (Table 1), was added to the sample instead of 2-propanol. With

Table 1
Data taken from Handbook of Chemistry and Physics, 60th ed., CRC Press, 1979–1980

Solvent	Dielectric constant at 20 °C	Dynamic viscosity at 22 °C (mPa s)
Water	80.2	0.95
Acetonitrile	37.5	0.39
2-Propanol	18.3	2.27
Tetrahydrofuran	7.4	0.47

tetrahydrofuran in the sample, the peak compression effect was also induced (Fig. 3B), indicating that the use of a solvent with a low dielectric constant is of high importance. Using 2-propanol or tetrahydrofuran in the sample also resulted in a temporary lowering of the currents, due to the effect on conductivity in the system.

Having established that peak compression was possible on the Chirobiotic V column, the next step was to optimize the system. In the pharmaceutical industry, it is often more relevant to examine enantiomeric purity (i.e. determine a low amount of the minor enantiomer) than to separate a racemic mixture. A drawback of the SCX–CEC system described before [7] was that analyte peaks often co-eluted when compressed, while they were separated under non-focusing conditions. Therefore, it was decided to aim to find conditions where peak compression occurred for only the minor enantiomer. By altering the concentration of acetonitrile in the mobile phase, it was possible to selectively cause peak compression for either one of the enantiomers of mianserin (Fig. 4). The system of 62.5% acetonitrile in the mobile phase was further investigated, as this system had the least baseline disturbances where the

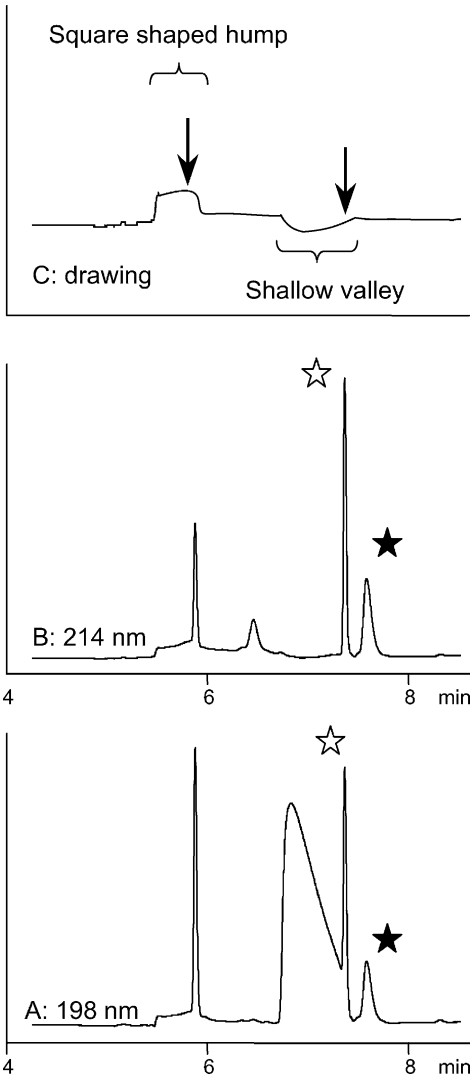

Fig. 3. (A and B) Electrochromatograms at different wavelengths showing that adding tetrahydrofuran to the sample also produces the peak compression effect. The shallow valley visible at 214 nm corresponds to a large peak at 198 nm originating from tetrahydrofuran. (C) Schematic drawing illustrating the “square-shaped hump” and the “shallow valley”. Sample: 208 μM mianserin dissolved in 30% tetrahydrofuran. Mobile phase: TEAA buffer, pH 4.8, 70% acetonitrile, ionic strength 9.4 mM. Electrokinetic injection 15 kV × 20 s; voltage 25 kV; current 6.0–7.0 μA.

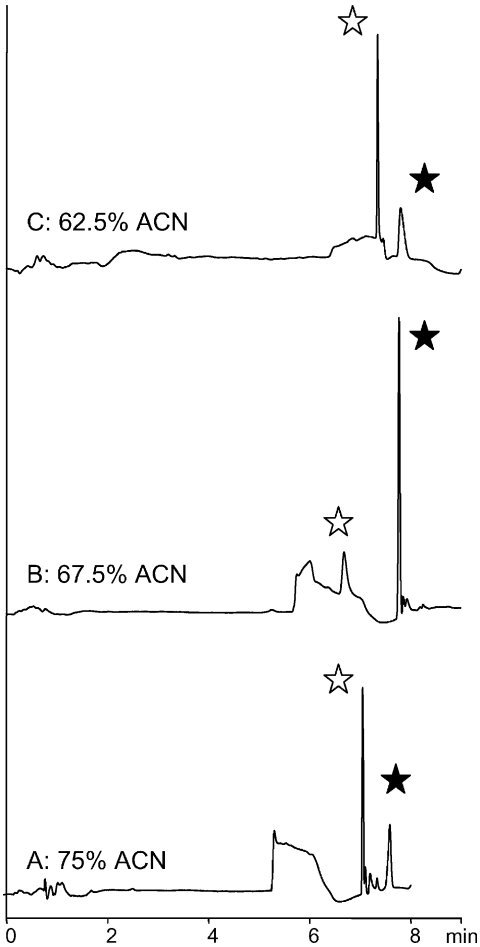

Fig. 4. Changing the concentration of acetonitrile in the mobile phase influenced which of the enantiomer peaks was compressed. Samples A and B: 63 μM mianserin dissolved in 30% 2-propanol. Sample C: 59 μM mianserin dissolved in 24% 2-propanol. Mobile phase: TEAA buffer, pH 4.8, acetonitrile content as denoted in the electrochromatograms, ionic strength 9.4 mM. Electrokinetic injection 10 kV × 15 s (A and B) and 15 kV × 25 s (C); voltage 25 kV; current 5.5–7.7 μA.

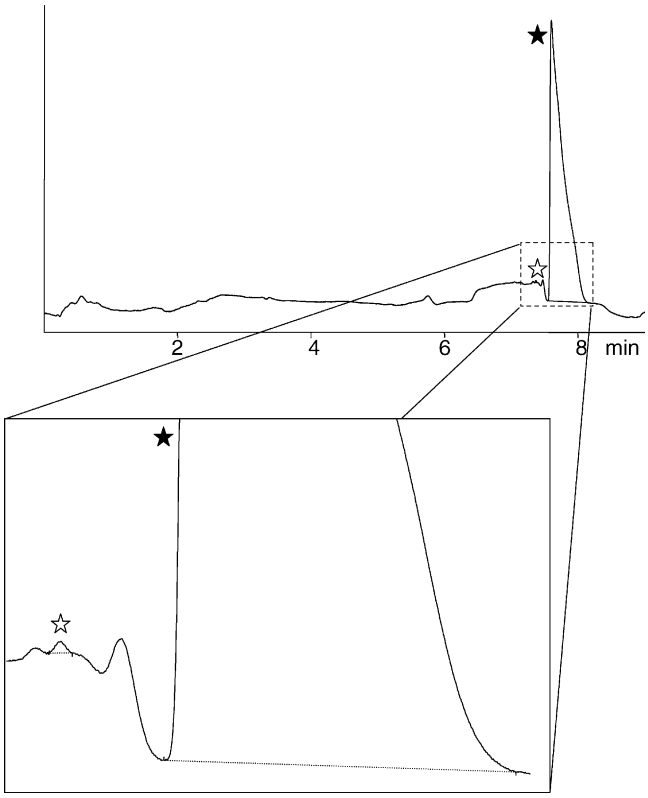

Fig. 5. Resulting electrochromatogram when analyzing mianserin where the minor enantiomer is present at a concentration of 0.07%. Sample: 250 μM mianserin dissolved in 25% 2-propanol. Mobile phase: TEAA buffer, pH 4.8, 62.5% acetonitrile, ionic strength 9.4 mM. Electrokinetic injection 15 kV × 25 s; voltage 25 kV; current 5.7 μA.

mianserin peaks eluted, and only the first eluting enantiomer peak was compressed. The concentration of 2-propanol in the sample also affected both the peak compression effect and the resolution. In the range tested (15–40%), the elution time of the first enantiomer was hardly affected, while, the second enantiomer eluted later with increased concentration of 2-propanol. A content of 25–30% 2-propanol in the sample gave the best conditions regarding resolution, peak shape and efficiency. When analyzing a sample of mianserin where the enantiomers were present as a 1:99 mixture, plate numbers of 1.4–1.6 million plates/m were seen for the compressed minor peak and around 50,000 for the non-focused (and probably slightly overloaded) major peak. By mixing appropriate proportions of the pure enantiomers and injecting them into the optimized system, the limit of quantification (LOQ) of the first eluting enantiomer was found to be 0.07% (Fig. 5). The LOQ was calculated as peak height 10 times the baseline noise level. The LOQ could not be further enhanced by using a higher sample concentration or larger injection volume since the major peak then became distorted and overlapped the minor peak. The corresponding LOQ under non-focusing conditions, where the minor peak had an efficiency of approximately 100,000 plates/m, was 0.7%. Hence, the peak compression effect resulted in a 10-fold improvement in sensitivity.

It is possible to discern a baseline pattern in Fig. 4 that occurs relative to the position of the sharp peaks. In Fig. 4C, the focused peak elutes at the end of a “square-shaped hump” and in Fig. 4A and B at the end of a “shallow valley”. This baseline pattern is illustrated in Fig. 3C. This indicates that there are at least two system zones where non-equilibrium conditions prevail and peak compression may occur on this type of column. As mentioned in Section 1, it has been shown in LC that due to the composition of the sample solvent, amine additives in the mobile phase can give rise to system zones that are connected to peak compression effects for basic analytes [6]. To evaluate the origin of the system zones on the Chirobiotic V column, we exchanged the triethylamine of the buffer for sodium hydroxide. With this mobile phase, peak compression was only seen in the shallow valley area. This suggests that peak compression in the square-shaped hump area is related to triethylamine in the mobile phase and the peak compression in the shallow valley area is related to the organic solvent in the sample. It is possible that the organic modifier in the sample solution alters the amount of triethylamine ions that are adsorbed onto the stationary phase, causing a zone with non-equilibrium conditions. Support for the argument that the second zone directly originated from the organic solvent was strengthened by the results obtained when using tetrahydrofuran in the sample. Here, the shallow valley, visible using UV detection at 214 nm, coincided with the tetrahydrofuran peak, visible using UV detection at 198 nm (Fig. 3A and B). Further support for this argument was obtained by adjusting the mobile phase compositions so that both peaks eluted between these zones when it could be seen that neither of the peaks were focused.

The observation that peak compression may occur within several system zones in the same electrochromatogram is highly interesting and possibly offers an explanation of some of the results previously reported in the scientific literature. In the original paper on peak compression in CEC, Smith and Evans published chromatograms of tricyclic antidepressants containing several peaks that were compressed but still well separated [1]. It is possible that this was due to multiple system zones initiated by the conditions used by these authors that resulted in peak compression of several sample components simultaneously at the boundaries of different non-equilibrium zones present in the CEC column.

In this study, as well as in the case in the SCX system [7], we observed that relatively large injection volumes were needed to focus the analytes. This is because the sample zone needs to be long enough for the analyte to be maintained within the sample induced system zone throughout the analysis so that continuous stacking at a boundary can occur without the analyte escaping. It was also observed that the compressed peak elutes later, relative to the non-focused enantiomer, resulting in reduced or increased enantioresolution. This implies that the mechanism behind the peak compression effect in this case is somewhat different to the previously proposed mechanism for SCX–CEC [7]. The positions

of the visible baseline disturbances in Fig. 3 in relation to the focused peaks indicate that peak compression occurred at the rear boundary of the system zones. This contradicts the mechanism presented previously and suggests that retention in the system zone is larger than in the surrounding mobile phase, thus forcing the analyte to the rear end of the system zone. Indeed, analysis with mobile phases containing 30% tetrahydrofuran or 2-propanol showed that retention factors were of the same magnitude as in mobile phases containing 80% acetonitrile. The higher retention is balanced by the increased electrophoretic velocity in the system zone due to the locally higher electric field, which prevents the analyte from escaping the system zone at the rear end. At the same time, the accelerating force of the electric field must not increase the electrophoretic mobility to such an extent that the focusing is lost. The fact that the effect on retention and electrophoretic velocity do not work in the same direction as in the SCX system might explain why the peak efficiencies are not increased in the Chirobiotic V system as much as in the SCX system.

4. Conclusions

It has been demonstrated that the peak compression effect can be utilized to improve quantification limits in chiral CEC. By adding solvents with a low dielectric constant, e.g. 2-propanol or tetrahydrofuran, to the sample solution, peak compression could be induced for the enantiomers of mianserin on a CEC column based on the chiral selector vancomycin (Chirobiotic V). This was obtained by adjusting the composition of the mobile phase so that the analyte eluted within either one of two system zones originating from the sample solution. Peak compression could selectively be induced to either one of the enantiomer peaks. A 10-fold improvement in the quantification limit for the minor enantiomer was obtained compared to elution under non-compressed conditions.

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