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Peak compression effects in capillary electrochromatography of basic drug substances using a strong cation-exchanger

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Abstract

Peak compression effects in capillary electrochromatography of basic drug substances using a strong cation-exchanger have been studied. Extremely narrow peaks with apparent efficiencies of several million plates per meter could be obtained when the composition of the sample zone differed from that of the mobile phase. The increased efficiencies were predominately observed when the analyte had an elution time similar to that of the electroosmotic flow marker. Peak compression was found to be reproducible and could be obtained for all investigated basic drug substances by altering the composition of the mobile phase in such a way that the analyte co-eluted with the sample zone. An explanation of the observed phenomena is proposed. A sample zone differing in composition from the mobile phase will disturb the equilibrium between the stationary and mobile phase. The elution rate of an analyte will consequently be different when residing inside the sample zone. If the analyte migrates through the sample zone at a higher speed than the rest of the mobile phase and is strongly retained after passing through a boundary in the sample zone, a continuous stacking can be obtained trapping the analyte as a very narrow band.

Keywords: Electrochromatography; Cation-exchangers; Peak compression; Pharmaceutical analysis; Buffer composition; Basic drugs

1. Introduction

Capillary electrochromatography (CEC) is a promising technique that can be regarded as a combination of liquid chromatography (LC) and capillary electrophoresis (CE) in which the high efficiency of CE is complemented with the vast possibilities of LC to affect the selectivity by means of both the stationary and mobile phase [1–6]. CEC is a potentially attractive option in pharmaceutical analysis as it could provide a solution to many demanding applications in the pharmaceutical field [7].

Smith and Evans introduced the use of a strong cation-exchanger (SCX) as stationary phase in CEC [8]. Remarkable results were obtained when analyzing a series of tricyclic antidepressants involving extremely compressed peaks with apparent efficiencies exceeding 8 million plates per meter. They concluded that the observed apparent efficiencies defied existing theory and that some form of sample stacking possibly was contributing to the mechanism behind this extreme peak sharpening effect. This paper obviously attracted much attention, but despite the efforts of several research groups the mechanism behind this focusing effect has not yet been fully explained [9–14].

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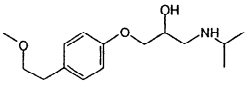
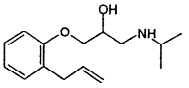
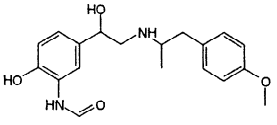
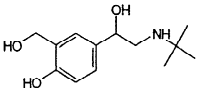
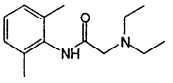
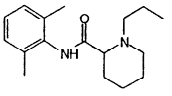
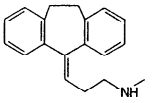
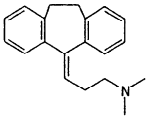
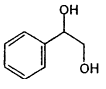
Euerby et al. observed apparent efficiencies of over 40 million plates per meter using a SCX column for the analysis of tricyclic antidepressants and proprietary basic compounds [10]. They reported, however, that the apparent efficiencies varied in an unexplainable way. The high efficiencies could be lost and severely fronting, tailing or split peaks were obtained for a period and then, for no apparent reason, high efficiencies were regained. The lack of reproducibility of the peak sharpening effect for analysis of tricyclic antidepressants has also been reported by Enlund et al. [7,11].

Spikmans et al. could not obtain the extremely high efficiencies reported by the other groups when analyzing tricyclic antidepressants on a SCX column using CEC coupled to mass spectrometry [12]. They suggested that the focusing effect does not originate from the column itself but from external effects.

The theory for zone migration of charged compounds in CEC has been reported by Ståhlberg [13]. This comprehensive paper offered a possible theoretical explanation for the narrow peaks obtained using SCX columns. It was shown that the combination of chromatographic and electrophoretic transport mechanisms give rise to nonlinear effects that may cause band broadening or, alternatively, a stabilized zone, which does not change its shape during the migration through the column. Under conditions where an analyte zone is stabilized by the influence of a zone with inhomogeneous field strength and a nonlinear adsorption isotherm, the peak shape of the solute will more or less be unchanged as it migrates through the column and band broadening due to other effects is thereby suppressed.

Chromatographic peak compression effects for analytes eluting close to system peaks caused by a different composition of the sample zone compared to the mobile phase have been reported. Nilsson and Westerlund studied sample induced peak compression of substituted benzamides in ion-pair reversed-phase LC [15]. Strode et al. observed high apparent plate numbers for clevidipine caused by the sample zone in supercritical fluid chromatography [16]. Moffat et al. reported reproducible apparent efficiencies up to 2.5 million plates per meter for analysis of partially anionic/neutral compounds using reversed-phase CEC [14]. The increased efficiencies were observed when the migration time of the analyte was closely matched to the elution time of sample-induced discontinuities in the mobile phase. An explanation was proposed in terms of nonequilibrium conditions caused by pulses of stronger or weaker solvent arising from a different composition of the sample zone compared to the mobile phase. These authors also discussed the even higher apparent efficiencies obtained by other research groups using SCX columns for the analysis of charged solutes.

Table 1
Structure, name and abbreviation of the analytes and the EOF markers

Structure	Name and abbreviation
	Metoprolol (Meto)
	Alprenolol (Alp)
	Formoterol (For)
	Salbutamol (Sal)
	Lidocaine (Lido)
	Ropivacaine (Ropi)
	Nortriptyline (Nor)
	Amitriptyline (Ami)
	1-Phenyl-1,2-ethanediol (PED)

They proposed that a possible reason for these results could be a twin sharpening mechanism caused by the sample induced system peak effect and an isotachophoretic effect.

In this paper, the peak compression effects for different basic drug substances in CEC using a SCX column have been studied.

2. Experimental

2.1. Chemicals

Most buffer stock solutions with pH between 2.8 and 7.2 were made from sodium hydroxide, phosphoric acid and water (HPLC grade). Other buffer components were both the acid and sodium salt of 3-(*N*-morpholino)propanesulfonic acid (MOPS) (Sigma, St. Louis, MO, USA), lithium hydroxide and potassium hydroxide. The ionic strength was calculated in all the buffer stock solutions [17]. Mobile phases were made from buffer stock solution, water and acetonitrile (gradient grade). The pH in the buffer was measured before addition of acetonitrile. Stock solutions of the drug substances (Table 1), metoprolol tartrate, alprenolol hydrochloride, formoterol fumarate, lidocaine hydrochloride, ropivacaine hydrochloride (AstraZeneca, Sweden) salbutamol sulfate, amitriptyline hydrochloride and nortriptyline hydrochloride (Sigma Chemical Co. St. Louis, MO, USA) were made from 1 to 3 mg of the substance dissolved and diluted to 5.0 ml with acetonitrile, except for formoterol fumarate and salbutamol sulfate that were dissolved in 1.0 ml of buffer stock solution (pH 2.8), 1.0 ml water and diluted to 5.0 ml with acetonitrile. 1-phenyl-1,2-ethanediol (PED) (Fluka, Buchs, Switzerland) was used as marker of the electroosmotic flow, and the stock solutions were 10 m*M* in water–acetonitrile (80:20) or 100% acetonitrile.

2.2. Instrumentation

The experiments were performed with fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, 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 210 nm, using 280 nm as reference, and sometimes 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 backpressure 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. The column was thermostated at 20 °C. The voltage was 20 kV (ramp 0.3 min) resulting in currents of 11–25 μA depending on the mobile phase.

2.3. Preparation of the stationary phases

The cation-exchange material was made from 3 μm bare silica GromSil particles (Grom, Herrenberg Germany) with a specific area of 100 m^2/g and a pore size of 300 Å. To prepare the material a three-step method was used described in detail in Ref. [11]. In short, the silanol groups of the silica were activated in nitric acid, silylated with (3-mercaptopropyl)trimethoxysilane and finally oxidized using in situ generated anhydrous trifluoroacetic acid peroxide, creating a propane sulfonate ion-exchange ligand.

2.4. Column preparation

The columns were packed according to the method described in detail by Enlund and Westerlund [18]. Sometimes the flow of the packing medium (acetonitrile–10 m*M* sodium chloride, 20:80 v/v) was very low and to ensure that sodium was present in the whole capillary when producing the frits, the slurry was made from 32 to 35 mg of stationary phase in 400 μl of a mixture of acetone–100 m*M* sodium chloride in water (90:10, v/v). The SCX stationary phase was, however, more difficult to pack than reversed-phase materials since the particles had a much higher tendency to aggregate. To minimize this effect, the slurry was ultrasonicated four times for 3 min with vortexing in between every sonication before introduction into the packing chamber. These

stationary phases also required higher temperature and longer (60 s) heating time to produce stable frits. Two columns were used in this study.

2.5. Evaluation of efficiency

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

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

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

3. Results and discussion

In order to investigate the cause of the peak compression effect, analyses of basic drug substances were performed where the pH, ionic strength, buffer components and acetonitrile content of the mobile phase as well as the length and composition of the sample zone were varied.

The effects of changing the composition of the mobile phase on the retention of the analytes were comparable to results reported earlier [11]. An increase in ionic strength resulted both in lower retention due to increased competition at the ion-exchange sites, and slightly decreased electroosmotic flow (EOF) due to decreased zeta-potential. Increasing the pH caused stronger retention and higher EOF, probably due to the dissociation of underivatized silanol groups, hence, more charges on the stationary phase. A higher content of acetonitrile resulted in reduced retention and lower EOF. The EOF was not affected using lithium or potassium as counter-ion instead of sodium in the phosphate buffer. Lithium is less retained than sodium in cation-exchange systems and, hence, the analytes where more retained compared to sodium phosphate buffer systems of equal ionic strength, due to less competition at the ion-exchange sites. The opposite effect was seen with potassium ions.

The length and composition of the sample zone were found to have a great influence on the peak compression effect. Fig. 1 shows electrochromatograms where a sample of ropivacaine was dissolved in mobile phase as well as in a sample solution differing in composition. As can be seen the solute eluted with approximately 100 000 plates per meter when using a rather large sample zone (100 kV s) and the sample dissolved in the mobile phase. However, a dramatic change occurred when the sample contained a higher percentage of acetonitrile than the mobile phase resulting in a very narrow peak with an apparent efficiency of almost 13 million plates per meter. In experiments using the same conditions, but introducing small sample zones, ropivacaine eluted with a normal peak shape and an efficiency of approximately 400 000 plates per meter independent of the composition of the sample zone. As long as the content of acetonitrile in the sample was higher than that of the mobile phase, sharp peaks could be produced, though, larger sample

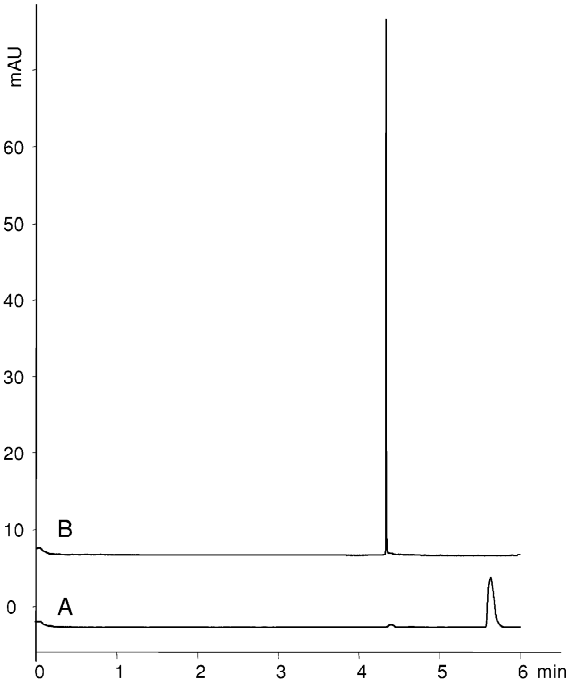


Fig. 1. The effect of the composition of the sample zone. A higher content of acetonitrile in the sample compared to the mobile phase generated compressed peaks, while equal amounts did not. Sample A: 34 μ*M* ropivacaine dissolved in mobile phase. Sample B: 34 μ*M* ropivacaine dissolved in sodium phosphate buffer pH 2.8, 76% acetonitrile, ionic strength 12.3 m*M*. Mobile phase: sodium phosphate buffer, pH 2.8, 60% acetonitrile, ionic strength 10.5 m*M*. Electrokinetic injection 100 kV s. Voltage 20 kV. Current 21 μA.

zones were needed when using a small excess of acetonitrile in the sample zone compared to higher amounts. Peak compression could also be induced by injecting a sufficiently large acetonitrile plug right after or in front of a sample dissolved in mobile phase. Neither the ionic strength of the sample, nor the amount of analyte introduced on the column seemed to affect peak compression. Small injections of very concentrated analyte did not result in highly efficient peaks, while large injections of very dilute samples did.

The elution rate of a positively charged compound in CEC is generally determined by the flow-rate of the mobile phase, the electric field strength, the electrophoretic mobility of the analyte and the interaction of the analyte with the stationary phase. The introduction of a sample zone differing in composition compared to the mobile phase will disturb the equilibrium between the stationary and mobile phase. A difference in conductivity between the sample zone and the mobile phase will also occur resulting in a different field strength within the sample zone compared to the mobile phase since the current is constant across the column [19–21]. The elution rate of an analyte will consequently be different when residing inside the sample zone compared to the rest of the mobile phase. The consequence of this is that the analyte will experience a change in elution rate when passing through the boundaries between the sample zone and the mobile phase. Depending on the interaction between the different factors that influence the elution rate, band broadening or peak compression may be obtained. Fig. 2 illustrates a case where the concentration of acetonitrile is higher in the sample zone than in the mobile phase, hence, the interaction of the analyte with the stationary phase will be lower and the electric field strength will be higher. The charged analyte, symbolized by (○), is transported through the sample zone at a higher speed than the rest of the mobile phase and is strongly retained after passing through the front boundary of the sample zone. Under these conditions, a continuous stacking can be obtained trapping the analyte as a very narrow

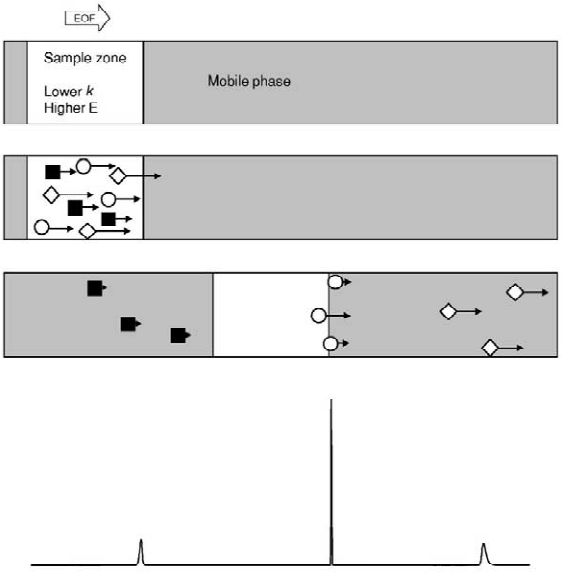


Fig. 2. Schematic representation of the peak compression effect and a resulting constructed electrochromatogram. *E*, Electric field strength, *k*, retention factor.

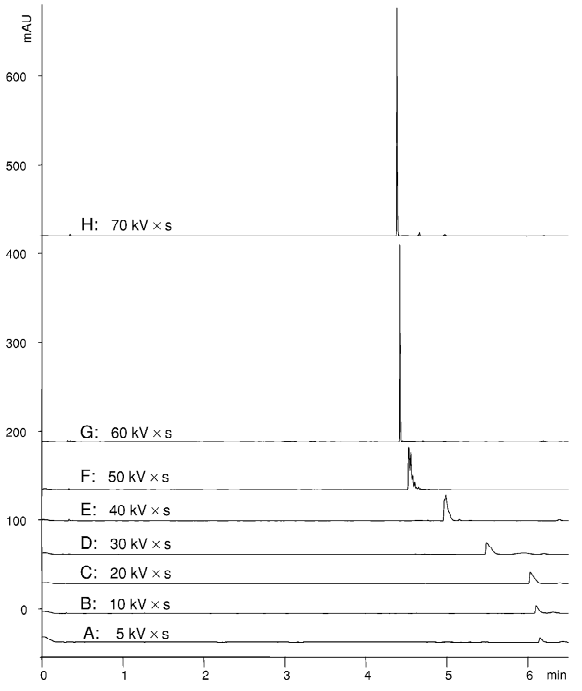


Fig. 3. The effect of the length of the sample zone. Electrokinetic injections as denoted in the electrochromatograms (A–H). Sample: 51 μ*M* ropivacaine in 88% acetonitrile, sodium phosphate buffer pH 2.8, ionic strength 0.8 m*M*. Mobile phase: sodium phosphate buffer, pH 2.8, 60% acetonitrile, ionic strength 10.5 m*M*. Voltage 20 kV. Current 20 μA.

band. The other two analytes are more (■) or less (◇) retained and are not caught by the sample zone and, hence, elute with normal peak efficiencies.

In Fig. 3 the effect of varying the length of the sample zone is demonstrated for peak compression of ropivacaine. Fig. 3A and B show electrochromatograms where the sample zone is kept small and consequently ropivacaine will be retained long

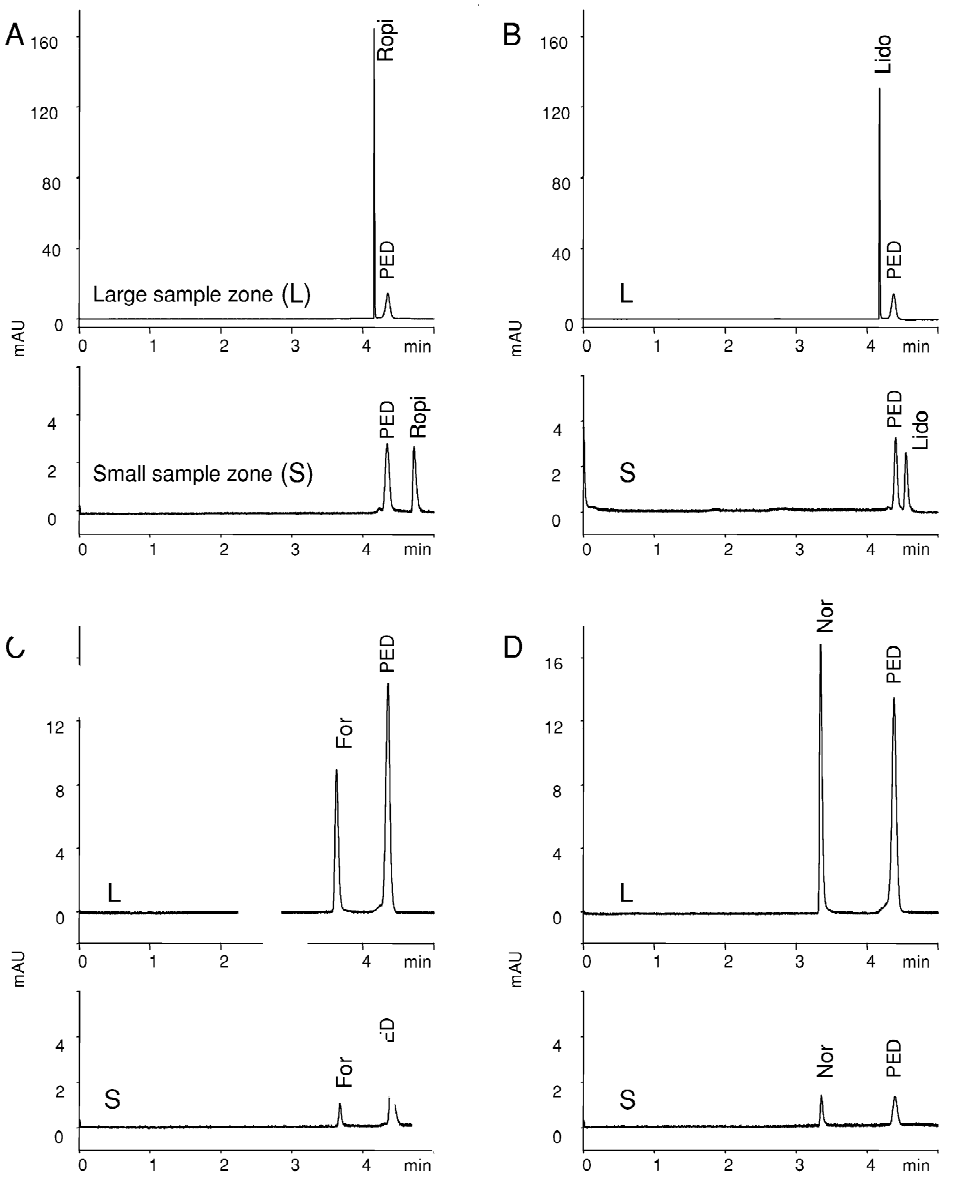


Fig. 4. Electrochromatograms of four drug substances under conditions causing and not causing peak compression. Peaks that are not focused when using a large injection (L) of 30 kV s elute at the same retention time as when using a small injection (S) of 5 kV s (C, D), while the retention for focused peaks is altered (A, B). Samples: sodium phosphate buffer pH 2.8, ionic strength 0.8 m*M*, content of acetonitrile 91% (A, B, D) and 87% (C). Concentrations of formoterol, lidocaine, ropivacaine and nortriptyline were 33, 56, 65 and 14 μ*M*, respectively, and the concentration of PED was 0.73 m*M* in all samples. Mobile phase: sodium phosphate buffer, pH 2.8, 60% acetonitrile, ionic strength 10.6 m*M*. Voltage 20 kV. Current 20 μA.

enough on the stationary phase in order to lag behind the sample zone in the initial state of the analysis and elute without the peak compression effect. The effect of increasing the length of the sample zone further resulted in the electrochromatograms in Fig. 3C–F. In these cases ropivacaine reside part of the analysis time in the sample zone and the rest of the analysis time lagging behind the sample zone resulting in broad and split peaks due to the difference in elution rate in the zones. Finally, Fig. 3G and H show electrochromatograms where the sample zone is long enough for ropivacaine to be maintained and continuously stacked within the sample zone throughout the entire analysis resulting in peak compression and high apparent efficiencies.

During the screening of different mobile phases the samples contained approximately 90% acetonitrile compared to 50–75% in the mobile phases. A small injection (5 kV s) was compared to a large one (30 kV s) and it was observed that peak compression generally was seen when the large sample zone was used. Fig. 4 shows the electrochromatograms of four drug substances injected under conditions causing and not causing peak focusing in one of the mobile phases. In this experiment lidocaine and ropivacaine showed strong peak compression while formoterol and nortriptyline eluted with normal efficiencies. As can be seen there is a retention shift for the focused peaks, which move close to the EOF marker when compressed, while the unfocused peaks remain at their initial position. This observation further supports the described mechanism involving a continuous stacking in the sample zone trapping the analyte at a boundary during transportation throughout the entire column resulting in a very high apparent efficiency. Fig. 5 displays the efficiency obtained when using a large injection plotted against the ratio of the elution times of the analyte and the EOF marker when using a small injection. The increased efficiencies were predominately observed when the conditions were such that the unfocused analyte eluted rather close to the EOF marker. Table 2 shows that peak compression of all analytes could be generated at some time by altering the composition of the mobile phase in such a way that the analyte eluted close to the EOF marker. Peak splitting was also seen, alone or in combination with peak compression.

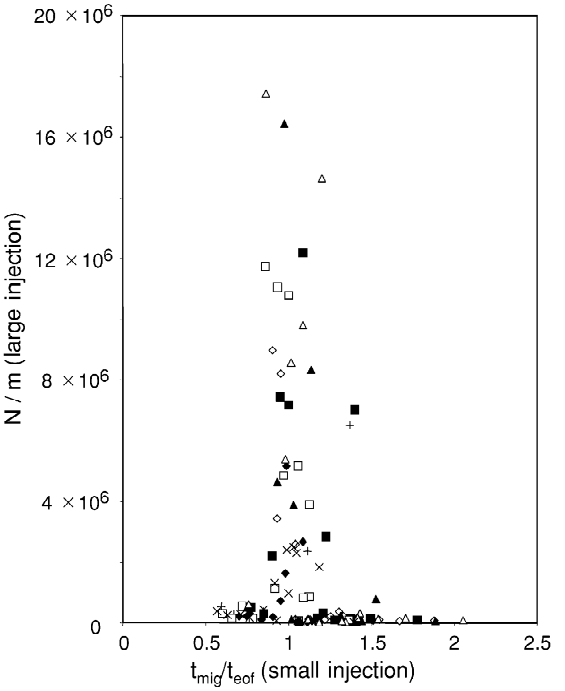


Fig. 5. The peak compression effect was predominately observed when the analyte (when non-compressed) had an elution time similar to that of the EOF marker. The ratio of the elution times of the analytes and EOF marker when using a small injection (5 kV s) plotted against the efficiency of the peaks when using a large injection (30 kV s). Sample concentration of metoprolol (■), alprenolol (□), formoterol (♦), salbutamol (◇), lidocaine (▲), ropivacaine (△), nortriptyline (×), and amitriptyline (+), were 40, 96, 33, 149, 56, 65, 14 and 27 μ*M*, respectively, dissolved in 90% acetonitrile, sodium phosphate buffer pH 2.8, ionic strength 0.8 m*M*. Mobile phases as in Table 2. Voltage 20 kV. Currents 11–25 μA depending on the mobile phase.

The reproducibility of the peak compression effect, caused by the sample zone, was found to be satisfying as can be seen in Table 3. As a result of the narrow peak width the detection limit of a focused compound will be greatly enhanced. The limit of detection of ropivacaine was improved from 33 μ*M* to 20 n*M* when comparing a 5-kV s injection of a sample dissolved in mobile phase containing 60% acetonitrile with a 80-kV s injection of a sample dissolved in 88% acetonitrile. Although the peak compression effect induced by the sample zone produces very narrow peaks with extremely high apparent efficiencies the retention window where these effects occur is limited and this might cause

Table 2
The effect of mobile phase composition on the peak shape of the analytes

Mobile phase composition																
pH	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	4.3	4.3	5.7	5.7	7.2	7.2	7.2[a]
Ionic strength (m*M*)	7.9	9.2	10.5	13.1	10.5	10.5	10.5	10.5	10.5	10.5	12.0	10.5	12.4	10.5	17.3	10.5
% ACN	60	60	60	60	50	55	65	70	75	60	60	60	60	60	60	60
Metoprolol			C	C	C	C	CS		C	S						C
Alprenolol	C	CS	CS	C	C	CS				CS		C	CS		C	CS
Formoterol					C	C				C						
Salbutamol				CS				CS	CS							
Lidocaine		S	C	CS	CS		CS	CS		S						C
Ropivacaine			C	CS	S	S	C	CS								C
Nortriptyline	C	CS			C	C				S	C	C				
Amitriptyline	C	C	CS		C	C				C		C				C

Electrokinetic injection 30 kV s of samples that had a higher acetonitrile content than the mobile phase. C = peak compression, S = peak splitting.

[a] Buffer made from MOPS, all other buffers are sodium phosphate based.

some limitations regarding the achievable resolution. This is exemplified in Fig. 6 showing electrochromatograms of the separation of six of the investigated drug substances. As can be seen in Fig. 6A, all six drug substances could be separated in a satisfactory way by injecting a small sample zone where only the two first drug substances, i.e. alprenolol and formoterol, are compressed. When increasing the injection volume, peak compression of all six drug substances was obtained (Fig. 6B). However, due to the retention shift, caused by the trapping of the analytes inside the sample zone, the resolution between the analytes was impaired. The analytes that elute after the EOF marker when using a small sample zone, all co-eluted when the large injection was used. Also note that the elution order between formoterol and amitriptyline is reversed.

The peak compression effect studied in this report was induced by the nonequilibrium conditions created by the introduction of a sample zone of a composition different from the mobile phase. The effect has proven to be reproducible and occurs within a limited retention window when the analyte has an elution time similar to the EOF marker. However, non-reproducible high efficiencies have also been reported for a peak with an elution time differing substantially from the EOF marker [7], moreover, electrochromatograms have been demonstrated with a number of focused peaks eluting over a time span of several minutes [8,10]. These observations have not been fully explained and may not be caused by the composition of the sample zone. It is possible that other effects, causing peak compression due to nonequilibrium conditions that are temporarily induced on the column, could explain these results.

Table 3
Reproducibility of apparent plate numbers per meter (N) and elution times (t) for 15 consecutive injections

	$N_{\text{alprenolol}}$	$t_{\text{alprenolol}}$ (min)	t_{PED} (min)
Average	4 866 800	4.31	4.76
RSD (%)	1.78	0.30	0.30

Sample: 52 μ*M* alprenolol dissolved in sodium-phosphate buffer pH 2.8, 88% acetonitrile, ionic strength 0.8 m*M*.

Electrokinetic injection 30 kV s. Mobile phase and conditions as in Fig. 5.

4. Conclusions

Extremely narrow peaks with reproducible apparent efficiencies of several million plates per meter (up to 17 million) have been obtained when the composition of the sample zone differed from the mobile phase and the analyte had an elution time similar to that of the EOF marker. In order to promote peak compression, the sample zone should be rather large and contain more acetonitrile than the mobile phase. The cause of this effect has been

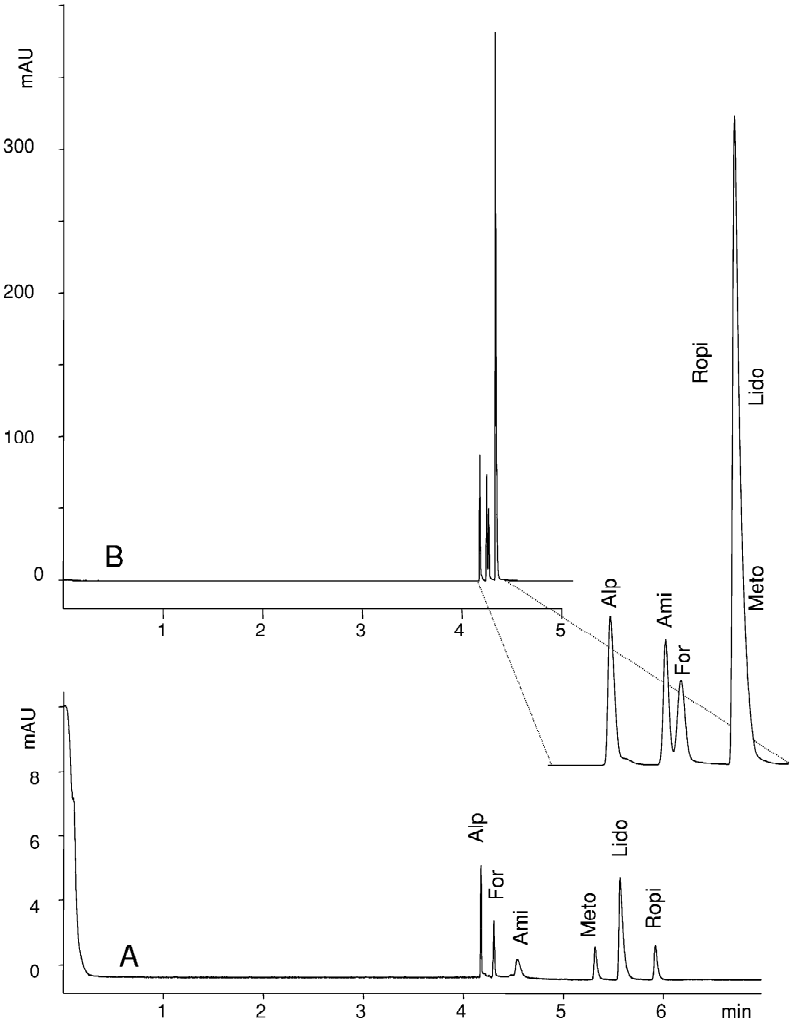


Fig. 6. Electrochromatograms showing the separation obtained for six drug substances under conditions causing and not causing peak compression. Resolution is impaired when peak compression is generated due to the retention shift caused by the trapping of the analytes inside the sample zone. Mobile phase: sodium phosphate buffer, pH 2.8, 60% acetonitrile, ionic strength 10.5 m*M*. Electrokinetic injections 5 kV s (A) and 40 kV s (B). Voltage 20 kV. Current 20 μA. Sample: metoprolol, alprenolol, formoterol, lidocaine, ropivacaine and amitriptyline 21, 25, 10, 67, 17 and 6 μ*M*, respectively dissolved in 88% acetonitrile, sodium phosphate buffer pH 2.8, ionic strength 0.8 m*M*.

explained by continuous stacking of the analyte in the sample zone trapping the solute as a very narrow band. The observed peak compression effect enhances detection limits but may cause some restrictions on the separating power of the CEC/SCX system due to the limited retention window where this effect occurs. In this paper basic drug substances with quite diverse chemical structures have been studied, therefore it is likely that also other types of positively charged organic analytes, eluting within this retention window, will be compressed on a SCX stationary phase. In the future we plan to examine whether similar effects can be produced for negatively charged analytes on an anion-exchanger.

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