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Enhancement of selectivity and concentration sensitivity in capillary zone electrophoresis by on-line coupling with column liquid chromatography and utilizing a double stacking procedure allowing for microliter injections

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Abstract

A double stacking procedure based on field enhancement is described as a means to increase the concentration sensitivity in capillary electrophoresis. Microliter volumes of sample are concentrated directly in the electrophoresis capillary without significant loss of separation performance. The whole procedure can be performed with a high degree of precision. A sensitivity gain of ca. 400 can easily be obtained by concentration of a 3-μl sample as compared with a 7-nl sample. The effect of experimental parameters on the final separation was studied for the enantiomers of *rac*-terbutaline, using alkyl substituted β-cyclodextrins as chiral selectors. Other enantiomers of chiral drugs can also be separated using the same procedure. Column liquid chromatography was used on-line with capillary electrophoresis for sample pre-treatment and concentration of the sample which allowed for injection of microliter volumes into the electrophoresis capillary. The selectivity and sensitivity gain of combining column liquid chromatography with capillary electrophoresis for determination of low concentrations of drugs in biosamples is exemplified.

1. Introduction

Although very high mass sensitivity detection is obtained in capillary zone electrophoresis (CZE), concentration sensitivity is poor. This is because the small diameter of the capillary only allows for small injection volumes (nanoliters). Thus determination of analytes at low concentrations e.g. drugs in biosamples, where concentrations in the range nmol–pmol/l are common [1], is hampered. Improved concentration sensitivity in CZE is therefore crucial in order to fully utilize the inherent potential of this technique.

There are a number of ways to increase the concentration sensitivity. For example by using more sensitive detection methods such as laser based fluorescence detection [2], electrochemical detection [3] and mass spectrometry [4]. UV–VIS detection is commonly employed in CZE but the concentration sensitivity which can be

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obtained by conventional approaches is poor, commonly μmoles/l. Several ways have been tried to improve on sensitivity using UV–VIS detection for example by using "z" cells [5] and other methods that increase the optical path length [6].

Another possibility to increase sensitivity is to utilize a sample pre-treatment step, preceding capillary electrophoresis. In this way selectivity can be gained as well as concentration of the sample from a larger sample volume and thereby more sample can be injected in the CZE capillary.

This will be of special importance when samples in complicated matrices are concentrated for further analysis by CZE.

Sample pre-treatment and pre-concentration can be performed either on- or off-line with CZE or directly in the CZE capillary. Several procedures have been proposed in the literature; some examples are the following.

1.1. On-line coupling with column liquid chromatography

Bushey and Jorgenson [7] showed that by combining CLC and CZE on-line, the combined system had a much greater resolving power and peak capacity than either of the two systems used independently of each other. Although not discussed by the authors, enhancement of concentration sensitivity could in principle also be obtained with their approach for example by injection of a preconcentrated sample before injection into the first column.

1.2. Solid-phase extraction

Solid-phase extraction is a commonly employed technique for sample pre-treatment of crude samples in order to isolate the analyte from matrix components. The technique allows for pre-concentration on-column [8,9] or either on- [10] or off-line [11] with CZE. Under favourable conditions up to 250-fold enrichment has been obtained for standard samples with the in-line plug extraction approach [8].

1.3. Isotachophoretic pre-concentration

Isotachophoresis (ITP), which is another mode of electrophoresis [12], has been used by several groups for on-line sample concentration with CE [13–15]. A part of the ITP zone is injected into the CE capillary and up to a 1000-fold enrichment has been reported [14]. Apart from the possibility of large volume injection using ITP, a gain in selectivity could also be obtained. A complication with ITP is that optimum analyte focusing demands that the electrolytes are properly chosen with respect to mobilities and pK values of the constituents and length of the stacking zones. Irreproducibility of the electroosmotic flow can also give rise to poor enrichment performance.

Recently several groups performed ITP-CE in a single capillary [16–19]. An increase in sensitivity by a factor 50 to 100 was obtained. This procedure can be carried out easily in commercially available instruments but still suffers from the difficulties in finding the right combination of buffer components. In one approach [17] a procedure was introduced that allowed for removal of all sample ions with mobilities below that of the analyte ion from the capillary column. The increased detectability and gain in selectivity makes this a promising method when dealing with e.g. biosamples. However, for trace analysis, sample pre-treatment might also be necessary in order to obtain sufficient selectivity [17].

1.4. Field enhanced pre-concentration (stacking)

An analyte dissolved in a sample matrix which has a lower conductivity than the background electrolyte will experience a locally increased field strength and higher electrophoretic mobility and will migrate with higher velocity than in the background electrolyte. When the analyte reaches the boundary between the sample matrix zone and the background electrolyte it will slow down again and stack into a zone much narrower

than the original sample zone. This field enhanced pre-concentration effect [20] can be utilized to increase the volume injected in CE [21]. The maximum volume (enrichment factor of about ten) at optimal conditions is restricted by the mismatch between the composition of the electrophoretic buffer and the sample plug [22]. A hydrostatic pressure (back-pressure) is created at both sides of the sample–background electrolyte boundary and the laminar back-flow causes additional band broadening.

Chien and Burgi showed that it is possible to increase the sample volume beyond the optimal conditions using both hydrodynamic [23] and electrokinetic injection [24]. In the first case they used the electroosmotic flow to remove the sample matrix zone during or after the enrichment step, before the start of the actual CE separation. The maximum sample volume which can be pre-concentrated without loss of any analytes out of the capillary end is shown to depend on the ratio of the electrophoretic mobility of the analytes to the electroosmotic mobility. For phenylthiohydantoin–aspartic acid anion, injection zones at least as long as 1/3 of the total capillary length could be injected without loss of analyte or much band broadening. In the second case Chien and Burgi utilized the field enhancement effect during electrokinetic injection. Enrichment factors up to several hundred could be obtained by replacing the buffer vial by sample vial during a reversed polarity step.

The performance of these field enhanced pre-concentration techniques as discussed by Chien and Burgi [25] was not investigated in detail. Recently Nielen [26] applied the techniques described by Burgi and Chien for pre-concentration of environmental samples. Nielen found that it was very difficult to obtain reproducible results utilizing the field enhanced electrokinetic injection procedure, but good reproducibility was obtained using field enhanced pre-concentration when 1/3 of the capillary was filled with weak buffer containing herbicides. As field enhanced pre-concentration require samples having a relatively low and reproducible conductivity it was necessary to pre-treat the environmental samples using membrane extraction disks.

1.5. Aim of present work

In earlier communications from this laboratory, results were presented from ongoing investigations on the use of CZE for bioanalytical work [27–29]. Apart from the need to increase sensitivity, it was emphasized that sample pre-treatment also will be necessary in order to get rid of matrix components which would allow for higher selectivity, to get a better match with the composition of the sample and the electrophoresis buffer, and to avoid contamination of the capillary. The approach we have taken to cope with the above needs is to use column liquid chromatography (CLC) for sample pre-treatment and pre-concentration on-line with CZE [29]. An analyte in a large sample volume e.g., one milliliter of plasma is concentrated into a smaller volume by for example solid-phase extraction and thereafter injected into a micro-CLC system (see Fig. 4). When the analyte elutes from the micro-CLC column (μl volumes), the whole volume is transferred to the CE capillary which can be partially or completely filled with sample. A double stacking procedure was then used for in-line concentration of the injected sample [28]. By using the double stacking procedure described below, almost complete filling of the electrophoresis capillary is possible without significant loss of CZE separation performance. In this way sample pre-treatment with a gain in selectivity, as well as a tremendous increase in sensitivity can be obtained. In our first investigations [29] we evaluated reversed-phase liquid chromatography using methanol as organic modifier because of the versatility of this separation mode for many compounds of biological interest.

In the present work the double stacking procedure was further evaluated for several experimental parameters using the enantiomers of the β-agonist terbutaline (cations) as model substances. The experimental conditions for the CZE separation of the enantiomers using alkyl

substituted β-cyclodextrins were reported earlier in a communication from this laboratory [30]. The potential of our multidimensional approach for bioanalysis is also demonstrated.

2. Experimental

2.1. Chemicals and buffers

2-Hydroxypropyl-β-cyclodextrin with an average degree of substitution of 0.9 and 2,3,6-"di"-methyl-β-cyclodextrin having the 2-, 3- and 6-hydroxyl groups partially substituted with methoxyl groups, the average degree of substitution being 1.8 was obtained from Wacker-Chemie.(Burghausen, Germany). 2,6-Dimethyl-β-cyclodextrin, β-cyclodextrin, *rac*-brompheniramine (maleate) and *rac*-ephedrine (hydrochloride) were obtained from Sigma (St. Louis, MO, USA), mesityl oxide from Aldrich (Steinheim, Germany), *rac*-terbutaline (sulphate) and *rac*-bambuterol (hydrochloride) from Astra Draco (Lund, Sweden) and *rac*-propranolol (hydrochloride) from Astra Hässle (Mölndal, Sweden). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

Phosphate buffer pH 2.5 (0.1 *M*) was prepared by mixing appropriate concentrations of phosphoric acid and sodium dihydrogenphosphate solutions. Phosphate buffer pH 7.5 (0.005 *M*) was prepared by mixing appropriate concentrations of sodium dihydrogenphosphate and disodium hydrogenphosphate solution.

All water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Mesityl oxide was used as neutral marker for measurement of electroosmotic flow.

Fig. 1 shows the structure of the analytes separated in this work.

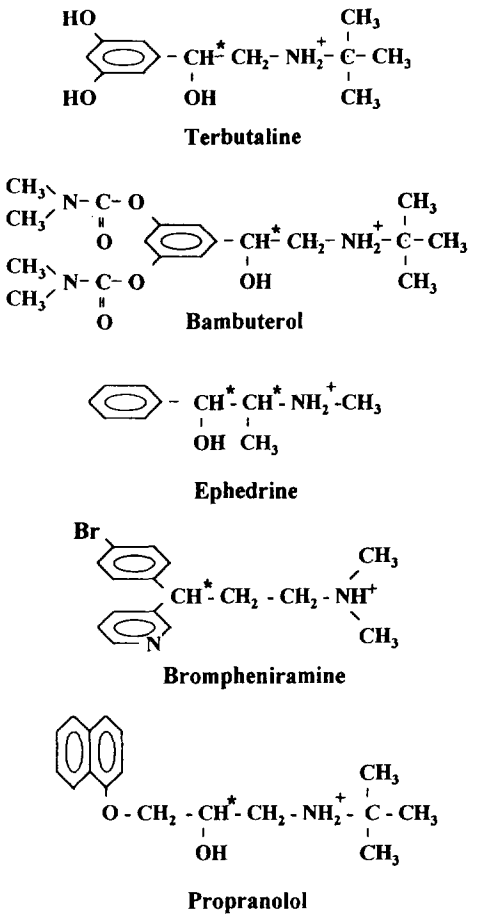

Fig. 1. Structures of chiral drugs separated in this work.

2.2. Double stacking procedure

The CE separations were carried out using a Prince (Lauerlabs, Emmen, Netherlands) programmable injection and high voltage power supply system. A variable wavelength UV absorbance detector (Model Spectra 100, Spectra-Physics, San Jose, CA, USA) equipped with a deuterium lamp was used for detection and operated at 210 nm. The separations were performed in 70-cm effective length (81 cm total length) untreated fused-silica capillaries, 75 μm I.D. and 372 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA). The separations were carried out at room temperature (ca. 23°C) and no forced cooling was applied to the capillary. Data analysis and collection were accomplished using System Gold software (Beckman, Palo Alto, CA, USA), version 712.

At the beginning of each working day the capillary was washed with 100 column volumes of 0.1 *M* NaOH solution. Before each run the capillary was washed with four column volumes each of 0.1 *M* NaOH, water and electrolyte

solution. After each working day the capillary was washed with ten volumes each of 0.1 *M* NaOH and water. The capillary was then dried with air.

Fig. 2 is a schematic representation of the different steps comprising the double stacking procedure. As the figure is only used to describe the different steps in the double stacking procedure mixing between zones during the procedure is not accounted for.

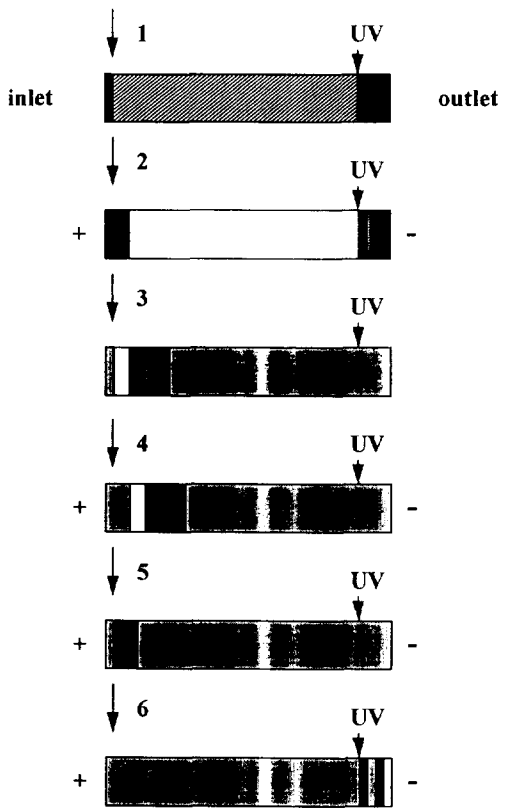

Fig. 2. A schematic presentation of the different steps comprising the double stacking procedure. Diagonal lines: 60% methanol–0.5 m*M* H_3PO_4–400 n*M* terbutaline; dark grey: 5 m*M* phosphate buffer pH 7.5; white: 60% methanol–phosphate buffer; black: terbutaline; light grey: 0.1 *M* phosphate buffer pH 2.5–10 m*M* dimethyl β-cyclodextrin. (1) Injection 200 mbar for 2.8 min; (2) stacking 30 kV for 6 min, back-pressure 44 mbar; (3) back-pressure 180 mbar for 1.8 min to move sample plug towards capillary inlet; (4) stacking 30 kV; (5) back-pressure 100 mbar to remove methanol plug, voltage lowered to 23 kV; (6) back-pressure off, final separation of terbutaline enantiomers.

During all stacking and separation steps a positive voltage was applied at the capillary inlet and the analyte cations migrated towards the cathode because of the net effect of the electrophoretic and electroosmotic mobilities.

The Prince system is equipped with a facility which allows for applying a back-pressure which is used in the different steps to either balance the electroosmotic flow or to pump the stacked band to the inlet part of the capillary.

In step one the capillary was filled (200 mbar for 2.8 min; 1 Bar = 10^5 Pa) almost to the detector window (3 μl) with sample solution (as marked by a distinctive change in the refractive index) exemplified here with a solution containing 400 n*M* *rac*-terbutaline (200 n*M* of each enantiomer), 0.5 m*M* H_3PO_4 and 60% methanol (pH 3.6). The bulk solution contained 5 m*M* phosphate buffer pH 7.5. The high pH of the bulk solution compared to the sample plug opposes the mismatch in electroosmotic flow at the boundary between the two zones reducing the laminar back-flow band broadening [22].

In step two the first stacking procedure was carried out by applying a voltage of 30 kV for 6 min, at the same time as a back-pressure of 44 mbar was applied. The back-pressure was used to prevent the stacking analyte from moving too fast towards the outlet of the capillary and allowing some of the first bulk electrolyte solution to remain in the capillary end. The back-pressure setting was judged to be right if the boundary between the methanol containing solution and the 5 m*M* phosphate buffer passed the detector at the end of the 6 min stacking (marked by a distinctive change in the refractive index as shown in Fig. 3). As almost all the field strength is dropped across the methanol containing solution, the electric field in the 5 m*M* phosphate buffer at the capillary outlet approaches zero. As a result the electrophoretic velocity of the ions in that buffer will approach zero and the ions will move only with the electroosmotic flow counteracted by the back-pressure of 44 mbar, thus no separation of the enantiomers will occur in this step.

In step three a separation buffer containing dimethyl-β-cyclodextrin as chiral selector was

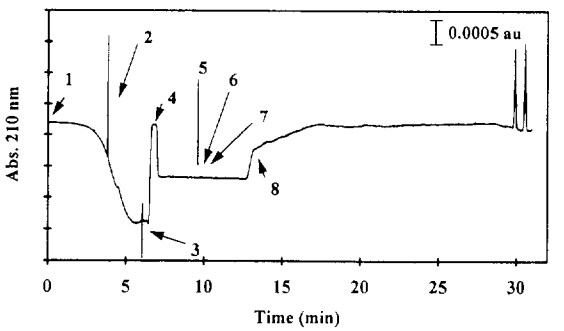

Fig. 3. Electropherogram obtained after using the double stacking procedure followed by CE enantiomer separation. 3 μl of 400 n*M* *rac*-terbutaline (200 n*M* of each enantiomer) was injected. Conditions as presented in Fig. 2. Arrows indicate the different events. (1) Stacking step one begins; (2) stacking peak of positive species; (3) voltage off, back-pressure on; (4) zone of 5 m*M* phosphate buffer pH 7.5; (5) back-pressure off; (6) voltage on, stacking step two at the inlet end of the capillary; (7) back-pressure on; (8) back-pressure off, final separation step begins.

introduced to allow for the separation of the terbutaline enantiomers [30]. At the same time as the 100 m*M* phosphate buffer pH 2.5 containing 10 m*M* dimethyl β-cyclodextrin was introduced the stacked peak was forced back to the injection end of the capillary. This was done by applying a back-pressure of 180 mbar to the system after turning off the high voltage. When about 3/4 of the original sample volume had been pushed out of the capillary which took 1.8 min (previously determined by injection of the 5 m*M* phosphate buffer) the back-pressure was stopped. The inlet end of the capillary was now filled with a mixture of the methanol solution, the stacked sample and buffer ions followed by a short band of the 5 m*M* phosphate buffer. The rest of the capillary was filled with the cyclodextrin separation electrolyte.

In steps four and five a second stacking procedure was carried out at 30 kV which compensated for the band broadening obtained after transportation of the previously stacked sample to the inlet part of the capillary. The positive species will now stack up at the concentration boundary between the 100 m*M* buffer containing cyclodextrin and the 5 m*M* buffer (mixed with methanol solution). The low pH of the cyclodextrin containing buffer (pH 2.5) compared to the 5 m*M* buffer (pH 7.5) will also increase the stacking effect in this step. At the same time as the stacking is in progress a back-pressure of 100 mbar is used to push the methanol containing band out of the capillary. After 2.5 min the separation voltage is lowered to 23 kV to prevent too much Joule heating in the system. The timing of this step was guided by the increase in current. During the first 2 to 3 min of the stacking the current in the system was low (1–3 μA) but it increased rapidly after that. When the current indicates that most of the high-resistant solution has been pushed out of the capillary (ca. 90 μA), the back-pressures was switched off. The final separation was obtained in step six.

Fig. 3 shows an electropherogram obtained with the double stacking procedure and enantiomer separation after injecting 3 μl of 400 n*M* *rac*-terbutaline (200 n*M* each enantiomer). Indicated in the figure is also the time for each event in the procedure. It is interesting to observe that the band broadening that has occurred after turning off the voltage and after pumping the sample back using back-pressure is compensated for in the second stacking step.

The information given above on experimental parameters that were used in the different steps are given as guidance. Differences from the experimental conditions given above are given in the text.

2.3. Micro-CLC on-line with CZE

In Fig. 4 the experimental set up is shown, which was used in this work to inject a sample corresponding to ca. 39 μl of plasma into the micro CLC column for further transfer (<3 μl) into the CE capillary. Before injection into the CLC column, the plasma sample (1 ml) was previously pre-treated and separated by coupled column liquid chromatography according to Edholm et al. [31]. After the last column [31], the fraction containing terbutaline was collected and transferred to a vial and evaporated to dryness and dissolved in 800 μl of water.

A pump (1) (Model 2150, Pharmacia LKB, Bromma, Sweden) was connected to an Acurate Microflow Processor (2) (LC Packings, Zurich,

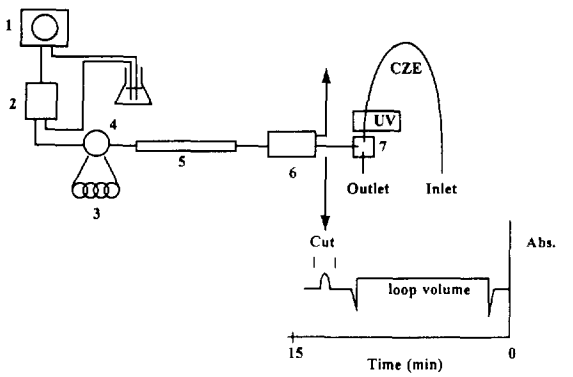

Fig. 4. Experimental set-up of micro-CLC coupled on line with CZE. (1) Pump; (2) flow processor; (3) loop; (4) valve; (5) analytical column; (6) μ-dumper interface; (7) Tee adapter. For details see the text.

Switzerland) resulting in a flow of 3 μl/min through the analytical column (5) which was made of steel (3.5 cm × 320 μm I.D.) and packed with Spherisorb ODS-2, 5 μm (LC Packings). A loop (3) with a volume of 31 μl connected to Valco valve (4) Model C6W KA 218 (Valco, Houston, TX, USA) was used for sample injection. To prevent bubble formation the mobile phase 80%, methanol–1 m*M* H_3PO_4, was degassed with helium.

The analyte fraction was transferred from the analytical column to the CZE capillary by switching of the μ-Dumper Interface (6) (LC Packings) a miniaturized switching device which was connected to the CZE capillary through a Fused Silica Tee Adapter (7) (Valco) having a inner diameter of 125 μm. The tee was positioned near the outlet of the CZE capillary which measured 90 cm from inlet to the detector, 95.5 cm from inlet to tee adaptor and 100.5 cm from inlet to outlet. While transferring the analyte fraction from the analytical column to the CZE capillary (previously filled with the CLC mobile phase) the outlet of the capillary was closed with a PVC stopper (manually operated).

Before starting the first step in the double stacking procedure the capillary was filled from the outlet to the detector (back-pressure 150 mbar for 0.9 min) with the 5 m*M* phosphate buffer (pH 7.5). Then the double stacking procedure was applied as described above.

3. Results and discussion

3.1. Performance of the double stacking procedure

In Fig. 5 the separation of *rac*-terbutaline (50 μM each enantiomer) after injection of 7 nl of a 100 μ*M* solution in 100 m*M* phosphate buffer pH 2.5 containing 10 m*M* dimethyl β-CD is compared to the injection of about 3000 nl (200 mbar, 2.8 min) 400 n*M* *rac*-terbutaline (200 n*M* each enantiomer) after using the above procedure. No considerable loss in separation performance is observed and the increase in sensitivity is almost 400 times (step two Fig 2, stacking for 6 min).

It was observed that the time used for stacking in step two (Fig. 2) of the stacking procedure produced differences in the recovery. Fig. 6 shows the effect of the time of stacking on the peak areas obtained in the final enantiomer separation. Stacking for 1 min contributes to more than 60% of the peak area obtained after stacking for 10 min. After 10 min of stacking a maximum in peak area has been reached.

This behaviour is reproducible and seems to have no practical consequences, except for lowering the sensitivity. At present we have made the following observations. In the first stacking step, a sharp or a broad peak can be obtained

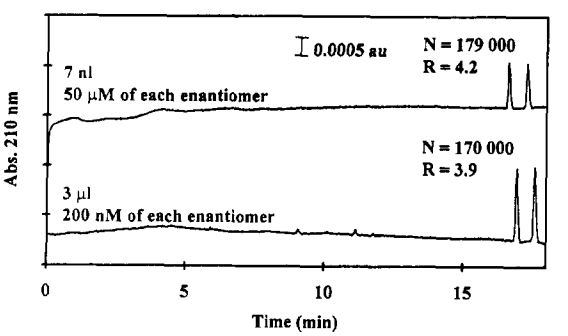

Fig. 5. Comparison of 7 nl injection versus 3000 nl injection. Conditions for 7 nl injection: 50 μ*M* each terbutaline enantiomer, 0.10 *M* phosphate buffer (pH 2.5) electrolyte, 2,3,6-"di"methyl β-cyclodextrin concentration 10 m*M* and separation voltage 23 kV; for 3000 nl injection: 200 n*M* each terbutaline enantiomer other conditions as presented in Fig. 2.

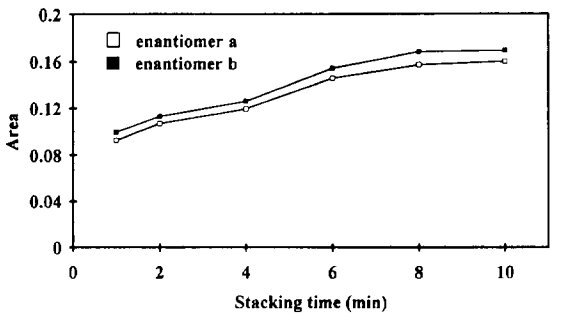

Fig. 6. The effect of the time of stacking on the peak areas obtained in the final separation. Conditions as presented in Fig. 2.

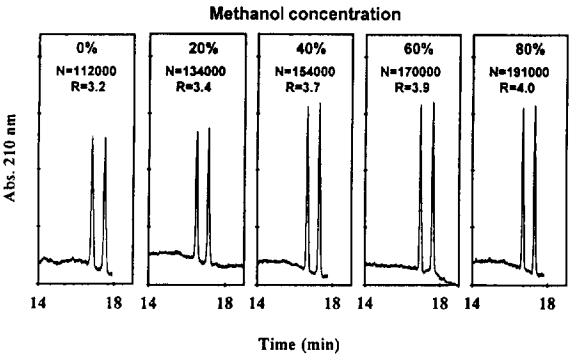

Fig. 7. Separation of terbutaline enantiomers using different methanol concentration (v/v) in the sample plug. Conditions as presented in Fig. 2 except for injection time, hydrodynamic back-pressure in the first stacking step and the time length of back-pressure in step 3 which were the following at each methanol concentration (v/v): 0% methanol: 2.1 min/ − 76 mbar/1.3 min; 20% methanol: 2.6 min/ − 68 mbar/1.7 min; 40% methanol: 2.8 min/ − 60 mbar/1.8 min; 60% methanol: 2.8 min/ − 44 mbar/1.8 min; 80% methanol: 2.4 min/ − 40 mbar/1.5 min.

independently of the stacking time but with no effect on the overall reproducibility. The low recovery we expect is due to loss of analyte in the step were the analyte is transferred back to the inlet. The high field strength at the beginning of the stacking step contributes to more than 60% of the recovery. As the field strength is lowered with time, the recovery will reach a plateau.

3.2. Effect of methanol and electrolyte concentration

The methanol concentration in the sample plug (Fig. 2, step one) affects the final enantiomer separation observed after step six. Fig. 7 shows the enantiomer separations obtained after the final step when the methanol concentration in the sample plug was varied from 0% to 80% keeping the buffer concentration constant (0.5 m*M* H_3PO_4). The injection and back-pressure times for the different solutions were adjusted according to the principles described under Experimental. Higher efficiency and resolution was observed at increased methanol concentrations. The stacking effect obtained in step two (Fig. 2) depends on the methanol concentration in the sample plug. Fig. 8 shows the electropherograms obtained during the first stacking step when the methanol concentration in the sample plug was 80% and 40%. A very large stacking effect was obtained at 80% methanol concentration but when the methanol concentration was 40%, 20% and 0% (as indicated by absence of a sharp peak) less stacking effect was observed. The field strength in the capillary will vary with the conductivity of the background electrolyte [32].

The introduction of methanol in the sample solution will increase the resistivity of the sample solution compared to an aqueous solution and

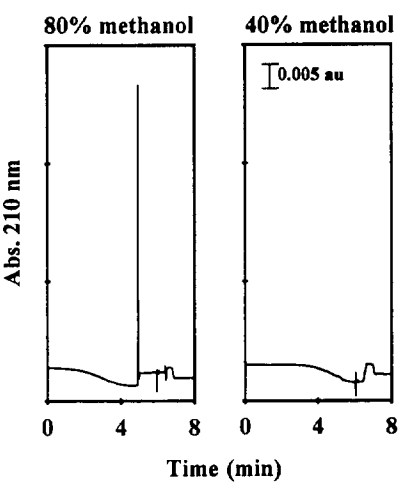

Fig. 8. Electropherograms showing the stacking effect in the first stacking step at methanol concentration (v/v) in the sample plug of 80% and 40%. Conditions as in Fig. 7.

thus the electric field strength in the sample zone will be raised further compared to the bulk solution and the stacking power increased, an effect which probably is the main reason why the efficiency and resolution is increased at higher concentrations of methanol. Fig. 7 demonstrates the importance of the second stacking step which compensates for the band broadening obtained in the preceding steps and thus makes it possible to handle large sample volumes. It can probably also be concluded, that the methanol concentration is of most importance in the second stacking step. The importance of the second stacking step is also clear from the fact that good reproducibility was obtained even though the effect of stacking varied from run to run in the first stacking step.

Fig. 9 shows the effect of ionic strength in the sample plug (Fig. 2, step one) on the final enantiomer separation at 60% and 80% methanol concentration when the double stacking procedure shown in Fig. 2 was followed (injection time and back-pressure times at 80% methanol were adapted to different viscosity). When the H_3PO_4 concentration in the sample plug is increased from 0.5 m*M* to 5 m*M* only a little loss of separation performance was observed at 80% methanol concentration, but at 60% methanol concentration the loss of separation performance was considerable. This observation also gives support to the above discussion on the importance of a high field strength. Higher methanol concentration in the sample plug thus allows for analysis of samples having higher ionic strength.

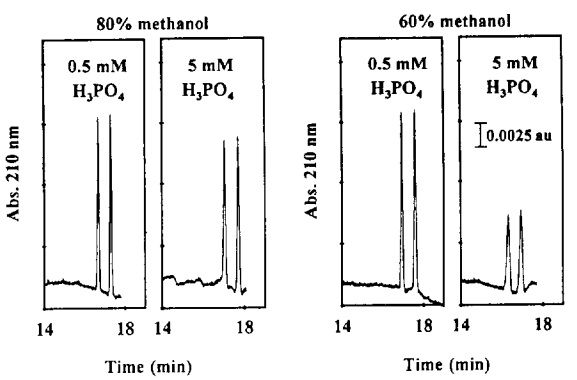

Fig. 9. Effect of electrolyte concentration in the sample plug on the final terbutaline enantiomer separation at 80% and 60% methanol concentration (v/v). Conditions as in Fig. 7 except for H_3PO_4 concentration.

In an untreated fused-silica capillary the electrophoretic migration and the bulk electroosmotic migration of ions depend linearly on the field strength.

Both the electrophoretic migration of sample ions and electroosmotic migration also depend on the composition, pH, concentration and viscosity of the background electrolyte. It has been observed that the mobility depends inversely on buffer concentration [33] and that organic solvents also affect the electroosmotic mobility dramatically [34,35].

Fig. 10 shows the effect of methanol concentration on the electroosmotic mobility in a fused-silica capillary, using mesityl oxide as neutral marker. The measurements were carried out in 0.5 m*M* phosphate buffer at pH around 3.5. It can be seen that the electroosmotic mobility starts to decrease with increased methanol concentration but reaches a minimum and starts to increase at higher concentrations. Also shown in the figure is the *relative* change in viscosity compared to pure aqueous buffer. The relative

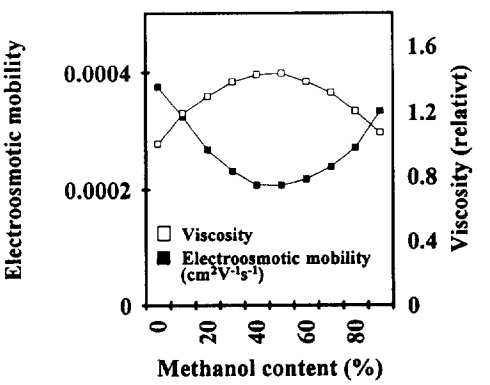

Fig. 10. Effect of methanol concentration (v/v) on electroosmotic mobility and relative viscosity of the electrolyte. Conditions; All solutions contained 0.5 m*M* H_3PO_4 giving the following pH values in the solutions. 0%, 10% and 20% methanol, pH 3.44; 30% methanol, pH 3.47; 40% methanol, pH 3.53; 50% methanol, pH 3.60; 60% methanol, pH 3.71; 70% methanol, pH 3.82; 80% and 90% methanol, pH 3.88. The neutral marker was mesityl oxide and voltage 30 kV.

viscosity was estimated from the time it took to fill 70 cm of 0.75 μm I.D. capillary to the detector window using 200 mbar hydrostatic pressure. The shape of the viscosity curve is in close resemblance, indicating the importance of the viscosity for the change of the electroosmotic flow at this low pH. As the charge at the silica surface is very much suppressed at this pH it would be plausible to conclude that the observed changes in electroosmotic flow are to a great extent due to the changes in viscosity. Other investigators [35] have shown that methanol suppresses the electroosmotic flow with increasing concentration (up to 80% methanol concentration). These observations were made at pH 9, so it is believed that the dependence found at pH around 3.5 as in this work will not be similar. At pH 9 the effect of viscosity will be less pronounced as compared to the effect on the charge on the wall of the capillary.

In order to obtain a good stacking effect in the first step it is important to reduce the laminar broadening due to mismatch in electroosmotic flow between sample zone and bulk solution [22]. As mentioned above, the high pH in the bulk solution compared to the sample plug (Fig. 2, step two) opposes the mismatch in electroosmotic flow. But the methanol concentration and ionic strength in the sample plug also affect the electroosmotic flow. At high compared to low methanol concentration the laminar broadening due to mismatch in electroosmotic flow seems to be more reduced. However any eventual mismatch in the first stacking step is compensated for in the second stacking step.

3.3. Linearity and reproducibility of the double stacking procedure

The linearity of the stacking procedure shown in Fig. 2 was studied for terbutaline enantiomer concentrations ranging from 12.5 n*M* to 500 n*M*. The calibration graph for peak areas is shown in Fig. 11. The results are the mean values from two runs. The correlation coefficient (r) for both enantiomers was 0.9998 and the intercept for enantiomer a was 0.0014 and for enantiomer b 0.0015.

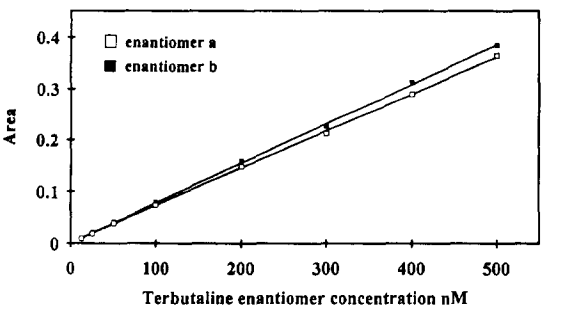

Fig. 11. Calibration graph at different concentrations of each enantiomer of *rac*-terbutaline. Conditions as presented in Fig. 2.

A study of the reproducibility of the double stacking procedure at different enantiomer concentrations is shown in Table 1. The results clearly indicate that sufficient performance can be obtained making the procedure most interesting for further evaluation in quantitative bioanalysis.

3.4. Application to other chiral analytes

Fig. 12 demonstrates the separation obtained after injection of 3 μl of sample solutions containing the enantiomers of some commonly used drugs, *rac*-bambuterol, *rac*-brompheniramine, *rac*-propranolol and *rac*-ephedrine in 80% methanol and 0.5 m*M* H_3PO_4.

After applying the double stacking procedure, the enantiomers were separated using the conditions previously described [30]. Baseline or almost baseline separation of all the enantiomers were obtained. Under the conditions used, all analytes were in their cationic form.

3.5. Column liquid chromatography on-line for sample pre-treatment and injection of microliter volumes into the CE capillary

For on-line coupling of CLC with CZE, micro columns were chosen because of the relative ruggedness of these columns and because μl-volumes of analyte eluting at reasonable reten-

Table 1
Reproducibility of the double stacking procedure at different concentrations

Parameter	Reproducibility (R.S.D., %)					
	Enantiomer a			Enantiomer b		
	1 μM ($n = 4$)	200 nM ($n = 7$)	62.5 nM ($n = 4$)	1 μM ($n = 4$)	200 nM ($n = 7$)	62.5 nM ($n = 4$)
Time	0.5	1.5	1.0	0.6	1.0	1.0
Peak area	0.7	1.2	2.4	1.0	0.9	3.5
Peak height	1.4	2.2	3.9	2.9	3.3	2.8
Resolution	1.9	1.7	1.1			
E (mean)	3.9	4.5	1.6			

tion times are compatible with the volume of an ordinary sized CZE column.

By connecting a micro CLC column directly to the CE capillary (Fig. 4), a cut [1] could be taken and transferred directly to the CE capillary. By this means sample pre-treatment as well as enhancement of concentration sensitivity can be obtained allowing for determination of low concentrations of analytes in biosamples. Fig. 13 shows an electropherogram, obtained after separation of a plasma sample containing the enantiomers of terbutaline at 5 nM concentration. The obtained electropherogram readily demonstrates the potential of the combined use of CLC for sample pre-treatment for enhancement of sensitivity and selectivity. Although terbutaline has a poor UV absorbance at λ 210 nm, nanomolar concentrations can still be reached.

The above results on the effect of methanol on the double stacking procedure shows that it

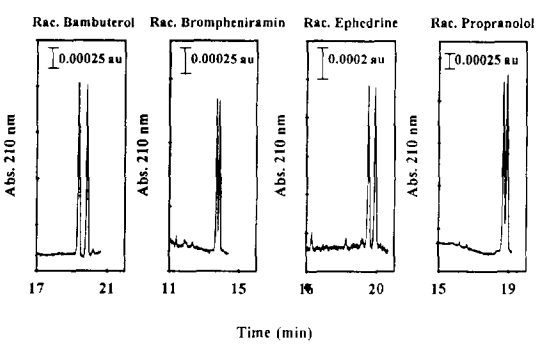

Fig. 12. Separation of the enantiomers of *rac*-bambuterol, *rac*-brompheniramine, *rac*-ephedrine and *rac*-propranolol after injection of ca. 3 μl sample. The sample plug contained 80% (v/v) methanol and conditions were as in Fig. 7 except for type and concentration of cyclodextrin in the final separation which were the following; *rac*-bambuterol–10 mM 2,6-dimethyl β-cyclodextrin, *rac*-brompheniramine–25 mM hydroxypropyl β-cyclodextrin, *rac*-ephedrine–25 mM 2,6-dimethyl β-cyclodextrin and *rac*-propranolol–10 mM hydroxypropyl β-cyclodextrin.

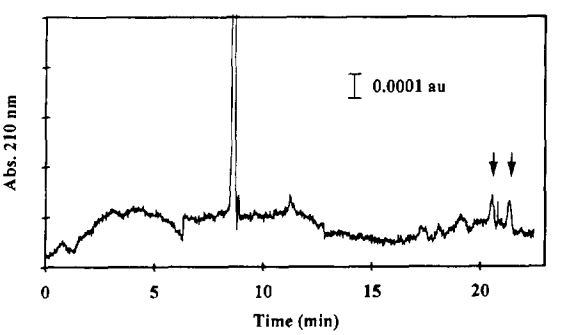

Fig. 13. Electropherogram obtained after the separation of a plasma sample containing the enantiomers of terbutaline at 5 nM concentration. The two arrows indicate both enantiomers. Conditions: CE capillary 90 cm to the detector, first stacking step 30 kV for 10 min using 51 mbar back-pressure, back-pressure in step three 180 mbar for 2.6 min, back-pressure in step five 150 mbar for 3 min then 100 mbar until current reached 90 μA. Second stacking step and the separation of terbutaline enantiomers were carried out at 30 kV. Other conditions see Fig. 2. For the experimental set-up see Fig. 4.

should be possible to use CLC mobile phases with high as well as low methanol content. Thus it should in principle be possible to apply the on-line approach to analytes of different lipophilicity.

4. Conclusion

The results presented in this work demonstrate that it is possible to concentrate μl-volumes of sample in a CZE capillary with mobile phase compositions commonly used in reversed-phase CLC without loss of separation performance which allows for an increase in concentration sensitivity. It is possible to carry out the double stacking procedure with good reproducibility by commercially available equipment. By combining CZE with CLC and sample preparation procedures like extraction using supported liquid membranes [36,37], large volumes (ml) of e.g. plasma can be pre-concentrated into microliter volumes which can be further concentrated by the double stacking procedure. The proposed procedure overcomes a significant problem in application of CZE to samples differing in composition and contamination of the CZE capillary is minimized.

Using different modes of liquid chromatography and other sample pre-treatment techniques, our approach should be investigated for application to other type of analytes in complicated matrixes. The concentration sensitivity which can be obtained is dramatic. Work is now in progress in our laboratory to explore our approach for quantitative analysis of drugs in biosamples using different combinations of sample pre-treatment techniques. Further work will also focus on more detailed investigations on the dependence of the double stacking procedure on experimental parameters.

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References

[1] L.-E. Edholm and L. Ögren, in I. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development*, Aster, Springfield, OR, 1986, pp. 345–373.
[2] P. Gozel, E. Gassmann, H. Michelsen and R.N. Zare, *Anal. Chem.*, 59 (1987) 44.
[3] R.A. Wallingford and A.G. Ewing, *Anal. Chem.*, 59 (1987) 1762.
[4] J.A. Olivares, N.T. Nguyen, C.R. Yonker and R.D. Smith, *Anal. Chem.*, 59 (1987) 1230.
[5] J.P. Chervet, R.E.J. van Soest and M. Ursem, *J. Chromatogr.*, 543 (1991) 439.
[6] S.F.Y. Li, *Capillary electrophoresis: Principles, Practice and Applications*, Elsevier, New York, 1992, pp. 55–154.
[7] M.M. Bushey, J.W. Jorgenson, *Anal. Chem.*, 62 (1990) 978.
[8] M. Merion, R.H. Aebersold and M. Fuchs, Poster presented at the *Third International Symposium on High Performance Capillary Electrophoresis*, San Diego, CA, February 3–6, 1991.
[9] I. Morita and J. Sawada, *J. Chromatogr.*, 641 (1993) 375.
[10] A.J.J. Debets, M. Mazereeuw, W.H. Voogt, D.J. Van Iperen, H. Lingeman, K.-P. Hupe and U.A.Th. Brinkman, *J. Chromatrogr.*, 608 (1992) 151.
[11] M.C. Roach, P. Gozel and R.N. Zare, *J. Chromatogr.*, 426 (1988) 129.
[12] A.T. Andrews, *Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications*, Oxford University Press, New York, 1987, p. 289.
[13] F.M. Everaerts, T.P.E.M. Verheggen and F.E.P. Mikkers, *J. Chromatogr.*, 169 (1979) 21.
[14] D.S. Stegehuis, U.R. Tjaden and J. van der Greef, *J. Chromatogr.*, 591 (1992) 341.
[15] F. Foret, E. Szoko and B.L. Karger, *J. Chromatogr.*, 608 (1992) 3.
[16] S. Hjertén, K. Elenbring, F. Kilár, J.-L. Liao, A.J.C. Chen, C.J. Siebert and M.-D. Zhu, *J. Chromatogr.*, 403 (1987) 47.
[17] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, *J. Chromatogr. A*, 653 (1993) 303.
[18] F. Foret, E. Szökö and B. Karger, *Electrophoresis*, 14 (1993) 417.
[19] C. Schwer, B. Gas, F. Lottspeich and E. Kenndler, *Anal. Chem.*, 65 (1993) 2108.
[20] S. Hjertén, S. Jerstedt and A. Tiselius, *Anal. Biochem.*, 11 (1965) 219.
[21] S.F.Y. Li, *Capillary electrophoresis: Principles, Practice and Applications*, Elsevier, New York, 1992, pp. 36–37.
[22] D.S. Burgi and R.-L. Chien, *Anal. Chem.*, 63 (1991) 2042.
[23] R.-L. Chien and D.S. Burgi, *Anal. Chem.*, 64 (1992) 1046.
[24] R.-L. Chien and D.S. Burgi, *J. Chromatogr.*, 559 (1991) 141.

[25] R.-L. Chien and D.S. Burgi, *Anal. Chem.*, 64 (1992) 489R.
[26] M.W.F. Nielen, *Trends Anal. Chem.*, 12 (1993) 345.
[27] S. Pálmarsdóttir and L.-E. Edholm, *Poster presented at the Fifth International Symposium on High Performance Capillary Electrophoresis, Orlando, Florida, January 25–28, 1993.*
[28] S. Pálmarsdóttir and L.-E. Edholm, Poster presented at the *13th Analytical Days, Lund, Sweden, June 14–18, 1993.*
[29] S. Pálmarsdóttir and L.-E. Edholm, Poster presented at the *Sixth International Symposium on High Performance Capillary Electrophoresis, San Diego, CA, January 31–February 3, 1994.*
[30] S. Pálmarsdóttir and L.-E. Edholm, *J. Chromatogr.*, 666 (1994) 337.
[31] L.-E. Edholm, B.-M. Kennedy and S. Bergquist, *Chromatographia*, 16 (1982) 341.
[32] M. Alonso and E.J. Finn, *Physics*, Addison-Wesley, Reading, MA, 1983, p. 478.
[33] H.J. Issaq, I.Z. Atamna, G.M. Muschik and G.M. Janini, *Chromatographia*, 32 (1991) 155.
[34] K. Salomon, D.S. Burgi and J.C. Helmer, *J. Chromatrogr.*, 559 (1991) 69.
[35] C. Schwer, and E. Kenndler, *Anal. Chem.*, 63 (1991) 1801.
[36] B. Lindegård, H. Björk, J.Å. Jönsson, L. Mathiasson and A.M. Olsson, *Anal. Chem.*, in press.
[37] B. Lindegård, S. Pálmarsdóttir, P. Deininger, L.-E. Edholm, L. Mathiasson and J.Å. Jönsson, in preparation.