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Electrically assisted capillary liquid chromatography using a silica monolithic column

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ABSTRACT

A silica monolithic capillary column was linked to an open capillary of the same internal diameter via a Teflon sleeve to form a duplex column to investigate the combination of chromatography and electrophoresis in the mode of electrically assisted capillary liquid chromatography (eCLC). Using a commercial CE instrument with an 8.5 cm long, 100 μm i.d. reversed phase silica monolithic section and a window 1.5 cm beyond the end of this in a 21.5 cm open section, a minimum plate height of 9 μm was obtained in capillary liquid chromatography (CLC) mode at a low driving pressure of 50 psi. In eCLC mode, high speed and high resolution separations of acidic and basic compounds were achieved with selectivity tuning based on the flexible combination of pressure (0–100 psi) and voltage. Taking advantage of the excellent permeability of silica monolithic columns, use of a step flow gradient enabled elution of compounds with different charge state.

1. Introduction

Improved control and fine tuning of the separation of charged samples may be obtained by adding an electrical field to pressure-driven capillary liquid chromatography (CLC), combining the two separation modes of liquid chromatography and capillary electrophoresis [1–4]. Researchers at Hewlett Packard (subsequently Agilent Technologies) [5–7] introduced the term electrically assisted capillary liquid chromatography, abbreviated as eCLC by Ivanov, Horváth and Karger [8], to describe this hybrid technique. Other variants of the name used by subsequent workers are electric field-assisted liquid chromatography [9–11], voltage-assisted liquid chromatography [12] and voltage-assisted micro-high performance liquid chromatography [13,14].

The critical difference between eCLC and pressure assisted capillary electrochromatography (pCEC) [15–22] is that electroosmotic flow (EOF) provides the dominant solvent flow in pCEC while pumping pressure is the only driver of the mobile phase flow in eCLC [7,8]. pCEC is normally operated at pH higher than 3 to enable a significant EOF on ionisable stationary phases. eCLC normally uses end-capped and base-deactivated chromatographic media, or silica-based phases without end-capping under suppressed mode (pH < 3) where EOF is nearly eliminated. In eCLC, the pressure-driven solvent flow across the microparticle packed capillary columns can be easily controlled and independently manipulated in either isocratic or gradient elution modes without any contribution or disturbance from EOF. The added electrical field only regulates the migration of charged compounds due to their electrophoretic mobilities and therefore provides a factor for selectivity tuning. The excellent and robust performance of eCLC is best exemplified in the arena of high selectivity and high resolution separations of peptide mixtures and protein digests [23–25]. Making comparisons with the relevant capillary scale separation modes of CEC and CLC, good promise has been demonstrated for this modified LC method [3,12].

Apart from the silica-based particulate material that has been widely used in pCEC and eCLC, monolithic materials have also been utilized in these hybrid electroseparation modes. Ericson and Hjertén [26] used a polyacrylamide based monolithic column for gradient separation of proteins in pCEC and eCLC. A solvent gradient generated using an HPLC pump was applied at one end of the capillary column to enhance the electrically driven separation of charged proteins. Polymethacrylate monolithic capillary columns were synthesized and used on home-built platforms to perform electrical field-assisted isocratic and gradient LC for the separation of model peptides and protein digests [8]. Hyphenated with ESI MS/MS, the potential utility of this separation strategy in proteomics research was demonstrated in the high throughput analysis of bovine serum albumin tryptic digest in less than 5 min with high sequence coverage [27]. Also for biological samples, silica-based monolithic capillary columns were used with the combination of gradient CLC and a dynamically changing electrical field (+30 to −30 kV) [28]. A commercial silica monolith was used in this study and the experimental system custom assem-

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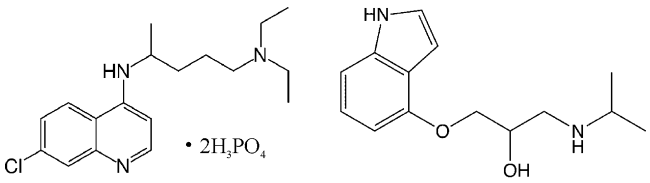

Chloroquine diphosphate salt Pindolol

Fig. 1. Molecular structures of pindolol and chloroquine diphosphate salt.

bled with a CE apparatus and HPLC gradient systems. Following the recognition and elucidation of the interplay of chromatography and electrophoresis in CEC [29,30], Euerby and coworkers investigated eCLC on a commercial CE instrument first with 3 and 12 μm particulate stationary phases for simultaneous separation of neutral, acidic and basic analytes [13] and later with capillary silica monolithic columns at low pH (2.7) [14].

A distinctive property of monolithic material is its high porosity [31,32]. With the silica-based monolithic capillary column as an example, porosities of up to 0.9 have been reported [31,33–36]. The high permeability enables reasonable flow rates to be obtained at low hydraulic pressure [34]. Two comprehensive reviews of monolithic columns for HPLC which include systematic comparisons to particulate stationary phases have recently been published [37,38]. In practice, the easy use of capillary silica monolithic columns driven by the very limited pressures (no higher than 12 bar) available in commercial CE instruments has been demonstrated in the modes of CLC [39], pCEC [36,40,41] and recently eCLC [14,42].

Using a continuous section of a commercially available monolith over the full length from input to output vials can require pressures higher than those provided by the CE system to allow elution in CLC in a reasonable time. For example, elution of the flow marker thiourea from a 30 cm Chromolith RP-18 column took 7 min at 44 bar [28]. In this paper, the aim is to extend the work presented in work by Liu et al. [39], where a duplex column consisting of a short monolithic section followed by an open section was used for fast separations by CLC or CEC. The objectives were to test the utility of a simple duplex column made by linking sections of monolith and open capillaries for separation and detection respectively, and to show the flexibility of eCLC separations for mixtures of negatively, positively charged and neutral small molecules.

2. Experimental

2.1. Materials and apparatus

An end-capped, C18 functionalised reversed phase capillary silica monolithic column of length 75 cm, 100 μm i.d./360 μm o.d., was provided by GL Sciences (Tokyo, Japan). The throughpore size, skeleton size and mesopore size were stated to be 2 μm, 1 μm and 15 nm, respectively, and the column porosity over 85%. UV transparent fused silica capillary 100 μm i.d./363 μm o.d (Polymicro TSU100375) used to make up the duplex columns was purchased from Composite Metal Services (Ilkley, UK). TFE Teflon tubing of 0.3 mm i.d./1.58 mm o.d., used as short sleeves for capillary coupling, was purchased from Sigma–Aldrich (Poole, UK). A capillary cutter was provided by GL Sciences (Tokyo, Japan) and used for fused silica capillary cutting. Sodium dihydrogen phosphate (for electrophoresis), phosphoric acid 85 wt.% in H_2O, sodium nitrate, thiourea, methyl-, ethyl-, propyl- and butylbenzenes of analytical grade were purchased from Sigma–Aldrich (Poole, UK). Two basic pharmaceutical compounds, pindolol and chloroquine diphosphate salt (Fig. 1) were donated by Dr Stephen Wren (AstraZeneca, Macclesfield, UK). Three acidic compounds, sodium 2-naphthalenesulfonate, disodium 1,5-naphthalenedisulfonate and trisodium 1,3,(6 or 7)-naphthalenetrisulfonate, were purchased from Sigma–Aldrich (Poole, UK). Acetonitrile (ACN) (HPLC grade) was purchased from Fisher Scientific (Loughborough, UK). Water was purified with an Elgastat UHQ II water purification system from Elga (Wycombe, UK). A manual syringe pump from Unimicro Technologies (Pleasanton, CA, USA) was used for column conditioning. The eCLC experiments were carried out on a Beckman Coulter P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) equipped with diode array detector (DAD). The system has a pressurization facility, which allows pressurisation of up to 100 psi. All samples were introduced by hydrodynamic injection, typically 5 or 10 psi for 10 s. The DAD detection range was set as 190–300 nm and data were displayed at wavelengths chosen within the range. Experiments reported in this paper were run without any coolant tubing and thermostating.

2.2. Fabrication of duplex column

To fit within the dimensions of the cartridge of the P/ACE MDQ instrument and allow short-end injection followed by detection in the open section as close as possible to the end of the monolith, the monolithic and open capillaries of matching i.d. and o.d. were cut to lengths 8.5 and 22 cm, respectively, and joined via a 1 cm long Teflon sleeve to make a duplex column with a total length of 30.5 cm. The detection point was set at 1.5 cm downstream from the interface of the two sections, i.e. 10 cm from the inlet end of the monolithic section. The duplex column arrangement reported in [39] also used a Teflon sleeve, but detection was carried out in the monolithic section. To make a good duplex column, the key step was creating flat cuts for the two ends to be butt jointed [43,44]. A reasonably good cut was obtained with the fused silica capillary cutter from GL Sciences. Otherwise, end grinding with a very fine grinding paper was necessary to secure a zero dead volume capillary connection. When grinding, the capillary was held perpendicular to the grinding paper placed on the bench. The ground end was regularly checked under a microscope to monitor flatness. When good flatness was obtained, the capillary was connected to a syringe to wash away the filings inevitably left inside the ground end. The two flat capillary ends were held together via a 1 cm long Teflon sleeve. An image of the butt joint within the sleeve is shown in Fig. 2, and the good alignment of the two capillaries and lack of dead volume is evident. The Teflon sleeve originally had a narrow bore of 0.3 mm, smaller than the o.d. of the fused silica capillaries (~360 μm). To adapt it as a capillary coupler, it was widened by forcing a fused silica capillary (363 μm o.d.) through for several cycles. In our experience, a freshly made Teflon sleeve should be utilized to make a robust butt joint. When the duplex column was assembled, it was connected to a manually driven syringe pump via

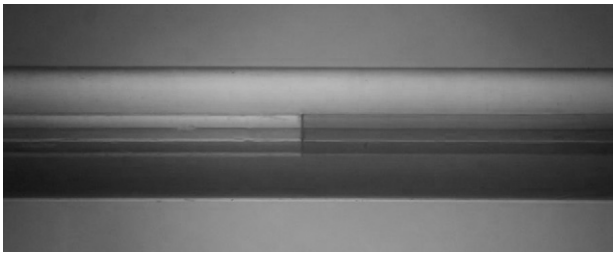

Fig. 2. Image of butt joint of duplex capillary silica monolithic column held in Teflon sleeve. Sleeve, 0.3 mm i.d./1.58 mm o.d., length 1 cm, initially widened by passing a 363 μm o.d. capillary through for several cycles. Monolithic section (right): 100 μm i.d./360 μm o.d., end-capped C18 reversed phase GL Sciences silica monolith, length 8.5 cm. Open section (left): 100 μm i.d./363 μm o.d., transparent fused silica capillary, length 22 cm (1.5 cm to detector).

Table 1
Separation performance comparison over 30 days on duplex column.

	Thiourea	Methylbenzene	Ethylbenzene	Propylbenzene	Butylbenzene
Retention time on 1st day (min)	1.28	1.48	1.54	1.65	1.80
Retention time on 30th day (min)	1.48	1.71	1.79	1.91	2.09
Retention factor on 1st day	–	0.16	0.20	0.29	0.41
Retention factor on 30th day	–	0.16	0.21	0.29	0.41
Peak efficiency on 1st day	–	–	–	9900	9300
Peak efficiency on 30th day	–	–	–	10,000	9700

CLC separation conditions: mobile phase ACN/H_2O, 80:20 (v/v); DAD at 200 nm; driving pressure 50 psi, 0.3 psi/5 s injection.

the monolithic end to equilibrate the chromatographic bed, prior to mounting into the cartridge of the CE instrument.

2.3. Electrically assisted capillary liquid chromatography

The duplex silica monolithic column was first investigated in capillary liquid chromatography (CLC) mode and pressures in the range 10–100 psi applied to provide the hydraulic flow. The mobile phase was ACN/H_2O, 80:20 (v/v). Alkylbenzenes (methyl-, ethyl-, propyl- and butylbenzenes) and thiourea (as dead time marker) were dissolved in the mobile phase at concentrations of 5.8, 5.1, 4.5, 4.0 and 3.9 mM, respectively. This sample solution was used for evaluating performance of the duplex column in CLC. Injection conditions were 0.3 psi for 5 s.

In eCLC experiments, both pressure (0–100 psi) and voltage (0–5 kV) were applied in various combinations. eCLC was first investigated for the separation of acidic compounds. The mobile phase was ACN/phosphate (50 mM, pH 7), 20:80 (v/v). The aqueous buffer was prepared with 50 mM NaH_2PO_4 adjusted to pH using 1 M NaOH. A mixture of sodium 2-naphthalenesulfonate, disodium 1,5-naphthalenedisulfonate and trisodium 1,3,(6 or 7)-naphthalenetrisulfonate was prepared by dissolving the components in the mobile phase at concentrations of 4.8, 2.1 and 0.9 mM, respectively, and loaded onto the column using pressure 10 psi for 10 s.

In the series of tests with basic compounds, the mobile phase was ACN/phosphate (10 mM, pH 2.3), 10:90 v/v. The aqueous buffer was prepared with 10 mM NaH_2PO_4 adjusted to pH using concentrated phosphoric acid. Although C18 functionalised silica monolithic columns are specified for use in the pH range 2.0–7.5, it should be noted that use of buffers with pH < 3 for extended periods is deleterious for stability and the column required flushing with aqueous acetonitrile after use with the acidic mobile phase. Basic pharmaceuticals, pindolol and chloroquine diphosphate salt were first dissolved in the aqueous phosphate buffer to obtain a stock solution of the analytes, respectively, as A, 1 mM chloroquine diphosphate salt plus 3.9 mM thiourea in 10 mM phosphate buffer at pH 2.3 and B, 3.2 mM pindolol plus 3.9 mM thiourea in 10 mM phosphate buffer at pH 2.3. Thiourea was included as a marker for the hydraulic flow in eCLC. The sample solution used for analysis was prepared by mixing 50 μL A, 50 μL B and 100 μL mobile phase, and injection was 5 psi for 10 s.

3. Results and discussion

3.1. Low-pressure capillary liquid chromatography

Previous publications [14,36,39–41] have shown that even driven by a very low pressure, reasonable flow rates can be obtained on capillary silica monolithic columns. Based on the pressurisation facility of the MDQ CE system, a series of low-pressure CLC separations of the alkylbenzene mixture was performed on the duplex capillary silica monolithic column using the mobile phase ACN/H_2O, 80:20 (v/v). Linear flow velocities were calculated knowing the length to the detector (10 cm) and the times of the flow marker thiourea. With 10–100 psi pressure, linear velocities in the range of 0.15–1.3 mm s^{-1} were achieved. This covers the optimum linear velocity for 3–5 μm porous particulate chromatographic media [45], the mainstream HPLC packing materials used over the past 20 years [46]. For butylbenzene, a minimum plate height of 9 μm was obtained at the flow rate of 0.7 mm s^{-1}, powered by a modest pressure of 50 psi (3.4 bar). Such a plate height value is characteristic of silica monoliths [38] and between those achievable under the best conditions on 3 μm and 5 μm particulate chromatographic media [38,45]. In addition, at 50 psi, the separation of four alkylbenzenes was completed within 3.5 min with a dead-time of 2.3 min. By contrast, an order of magnitude higher pressure (500–600 psi) is usually needed to achieve the same separation performance on particulate phases [31,34,47,48]. In this sense, the CLC separation on the silica monolithic column can be treated as low-pressure high performance LC. An advantage of using the duplex 8.5 cm monolith plus 21.5 open capillary rather than a single 30 cm monolithic capillary column is that the optimum flow rate could not have been reached with the latter. Since driving pressure for a fixed flow rate scales linearly with column length, the pressure required would have exceeded the 100 psi maximum available with the Beckman Coulter CE instrument.

To investigate column robustness, the same duplex silica monolithic column was used throughout an extended series of CLC/eCLC experiments. The column performance was monitored before and after 30 days of use with basic analytes in acidic media (see Section 3.2) via CLC separations of the alkylbenzene mixture and the results are shown in Table 1. Although there was some lengthening of the elution time the performance of the duplex capillary silica monolithic column was excellent in terms of the reproducibilities of retention factor and peak efficiencies.

3.2. Electrically assisted capillary liquid chromatography

A mixture of anionic species (all as sodium salts): nitrate, 2-naphthalenesulfonate, 1,5-naphthalenedisulfonate, 1,3,(6 or 7)-naphthalenetrisulfonate, and a neutral, thiourea, was analysed at neutral pH, as shown in Fig. 3. In the CLC mode (Fig. 3A), only 2-naphthalenesulfonate (peak 4) was retained even with a low organic concentration of 20% ACN, whilst others co-eluted with thiourea at the dead time. Tailing of peaks of charged analytes, as seen with peak 4, has previously been noted in LC on C18 functionalised silica monolithic columns [49]. Since 2-naphthalenesulfonate was injected at a relatively high concentration (4.8 mM), peak tailing could be due to overload effects from multiple adsorption sites and/or adsorbate–adsorbate repulsion effects [50]. When an electrical field of 5 kV was applied over the duplex column, the separation was significantly influenced (Fig. 3B–D). Comparing Figs. 3A and B, the slight decrease in time for the flow marker species thiourea (peak 5) on applying voltage is attributed to Joule heating, which decreases solvent viscosity and increases flow velocity at constant pressure. Reducing the pressure from 100 to 30 psi while keeping the voltage at 5 kV, the overall resolution of the analytes was improved. The optimum result was obtained at 5 kV +30 psi (Fig. 3D), with baseline resolution between

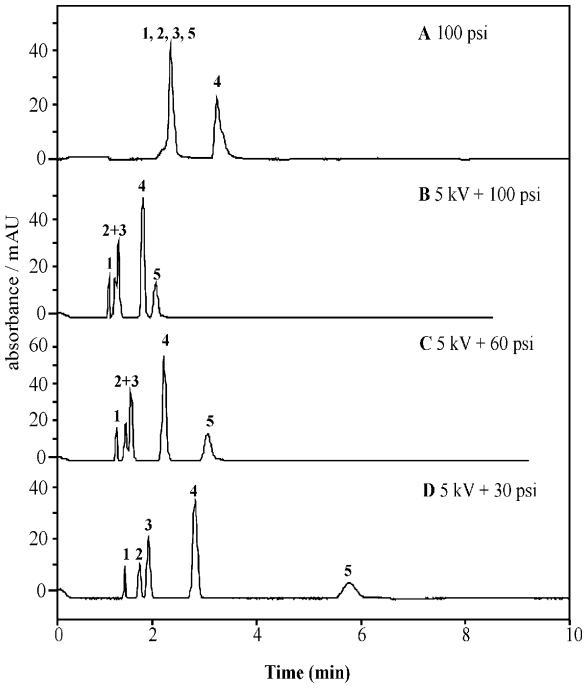

Fig. 3. CLC/eCLC separation of acidic sample: duplex capillary silica monolithic column; mobile phase ACN/phosphate (50 mM, pH 7), 20:80 (v/v); DAD at 220 nm; 10 psi/10 s injection from short end; separation conditions as labelled in the figure, (A) is in CLC mode and (B–D) are in eCLC mode at reversed polarity (i.e. cathode at the inlet end). Analytes are 1: nitrate; 2: 1,3,(6 or 7)-naphthalenetrisulfonate; 3: 1,5-naphthalenedisulfonate; 4: 2-naphthalenesulfonate; 5: thiourea.

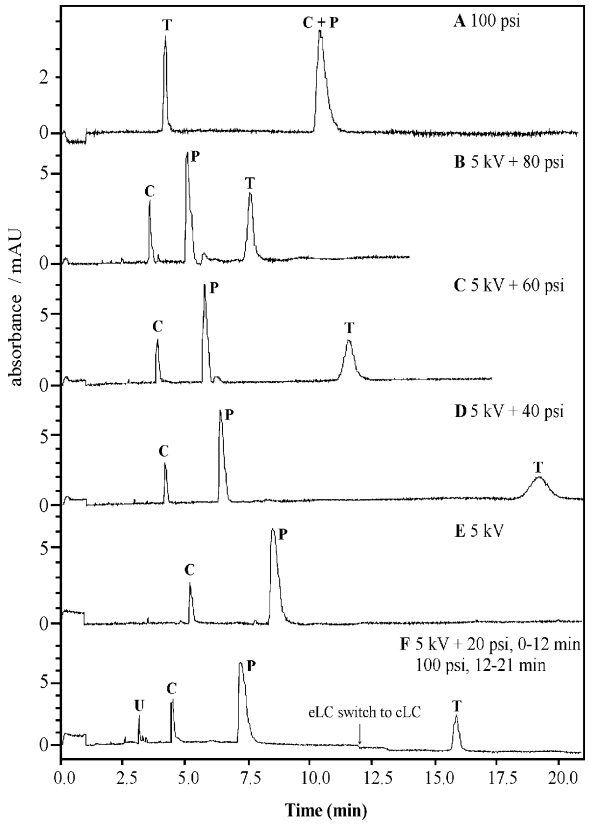

Fig. 4. CLC/eCLC separation of basic sample: duplex capillary silica monolithic column; mobile phase ACN/phosphate (10 mM, pH 2.3), 10:90 (v/v); DAD at 215 nm; 5 psi/10 s injection from short end; separation conditions as labelled in the figure, (A) is in CLC mode and (B–F) are in eCLC mode at normal polarity (i.e. anode at the inlet end). Analytes are C, chloroquine; P, pindolol; T, thiourea and U, unknown peak.

all analytes and the neutral marker peak visible. For the ionic analytes, improvement in resolution with decreasing pressure is due to the greater time over which the analytes experience the electric field during their passage to the detector.

Fig. 3B–D shows that separation selectivity in eCLC for this set of weakly or non-retained negatively charged species is determined by the charge to size ratio. The high electrophoretic mobility of the nitrate ion, arising from the negative charge and very small molecular size, caused it to be the first peak to elute in the eCLC separations, with peaks 2, 3 and 4 being the tri-, di- and mono-naphthalenesulfonates. This accords with work by Euerby et al. [14], where the elution order of weakly retained acidic analytes in eCLC was found to be almost solely due to their differential electrophoretic mobilities. It is important to note from Fig. 3B–D that there is no peak distortion due to Taylor dispersion in any of the peaks, showing that the combination of pressure and electrically driven flow in the 1.5 cm length to the detector post-monolith does not affect peak shapes of the neutral and charged species. This shows that the duplex approach provides a simple way to use a silica monolith column for eCLC in a Beckman Coulter CE instrument, using a short Teflon sleeve as in [39] to encase the joint to an open capillary of the same i.d. and o.d. An additional benefit of having an open capillary with UV transparent coating for post column detection is that there is no need to strip any cladding off either section to create a window, making this approach easier to implement by comparison with previous work [39].

A mixture of two basic pharmaceuticals, chloroquine and pindolol, together with a neutral thiourea were also studied in eCLC using the 8.5 cm long silica monolithic column and mobile phase ACN/phosphate (10 mM, pH 2.3) 10:90 (v/v). Results are shown in Fig. 4. In CLC mode, i.e. in the absence of an electric field, chloroquine and pindolol co-elute (Fig. 4A). Peak tailing of basic species as seen here has previously been observed in LC on C18 functionalised silica monolithic columns, e.g. with 0.8 mM propranolol [49]. The concentrations of chloroquine and pindolol in the injected sample solution were 0.25 and 0.8 mM, respectively, and as discussed in the case of the anions peak tailing could be due to overload effects from multiple adsorption sites and/or adsorbate–adsorbate repulsion effects [50]. In eCLC mode (Fig. 4B–F), baseline resolution is readily achieved over a wide range of pressures, with the dicationic species chloroquine eluting before the monocation pindolol as expected. The concentration of the Na^+ co-ion in the mobile phase was 9 mM, and whilst this low value means that there is very little Joule heating in eCLC it does lead to additional peak asymmetry due to electromigration dispersion. In the range 0–60 psi (Fig. 4C–E), relatively constant resolution was obtained: values calculated from peak widths and times were 5.6, 5.6 and 5.4 at pressures 60, 40 and 0 psi, respectively. Resolution decreased to 3.8 at 80 psi. Peak efficiencies (chloroquine) in excess of 10^4 were obtained. This shows that pressures up to 60 psi do not degrade resolution and do not significantly affect peak efficiency, while offering the benefit of reducing the migration time for the late eluting analyte (pindolol) from 8.4 to 5.8 min. Additionally, it is worth noting that when the mixture involves neutral species, application of pressure is necessary to secure elution of all the components of different charge states in the sample. As evidenced in Fig. 4E (5 kV + 0 psi), the neutral analyte, thiourea, did not elute within the time range of 0–20 min. Since the flow rate can be easily regulated by simply increasing or decreasing the pressure [31,33–36,47], the duplex silica monolithic column can be used in step flow gradient operation [48]. Fig. 4F shows the effect of switching off the voltage and stepping up the hydraulic pressure from 20 to 100 psi after elution of the second charged analyte. The step change was made at 12 min, and the peak from thiourea is seen at 16 min. It is evident

that when working in eCLC mode using silica monolithic columns, step changes in solvent flow rate can be readily implemented to reduce any vacant elution window and speed up the separation accordingly.

By contrast to use of monoliths, previous pCEC/eCLC work with microparticle packed columns has shown that to obtain acceptable solvent flow rates, use of HPLC pumps was normally necessary to supply the required high pressure [1–4,18–22]. In a study comparing CLC and pCEC/eCLC on a 5 μm particle packed column, initial work was carried out using a commercially available CE instrument (HP^{3D} CE) with maximum pressure 12 bar [51]. However, this instrument could not deliver the required flow rates for the target separations using mobile phase ACN/aqueous ammonium formate 50:50 (v/v), and subsequent work required a setup including an HPLC pump which allowed for pressure drive of up to 60 bar [51].

In agreement with other reports [14,39,41], the current results shows that silica monoliths can be an ideal column material to enable simple eCLC operation on a CE platform. As noted in Section 3.1, use of a duplex arrangement and short-end injection [39] rather than a single long monolithic capillary has the advantage of allowing plate height contributions from pressure-driven flow to be optimised when using a CE instrument with a limited working pressure range. This consideration is particularly important with mobile phases of high aqueous content. For example, the viscosity for acetonitrile/water mixtures [52] of composition ACN/H_2O 10:90 and 20:80 (v/v) is twice that of 80: 20 (v/v) mix discussed in Section 3.1, so optimum pressures are correspondingly higher and separations would be unacceptably long without short-end injection.

4. Conclusions

The potential for flexible operation of silica monolithic capillary columns was explored in the mode of electrically assisted capillary liquid chromatography, using a duplex column configuration with an 8.5 cm long silica monolith linked to a transparent open capillary to allow post column detection without the need for stripping capillary coating to create a window. The low backpressure of the monolith enabled hydrodynamic injection and high performance LC to be performed using a commercial CE system operating over a modest pressure range, here a Beckman Coulter P/ACE MDQ with pressures 0–100 psi (6.9 bar). Separations for negatively, positively charged and neutral compounds are reported in eCLC mode, in which pressure and electrical field were combined together to manipulate the separation speed and selectivity independently in a simple, straightforward and effective way. The experimental results have proved that eCLC can achieve peak efficiencies in excess of 10^4 on columns of length 8.5 cm for charged small molecules, which is at a comparable level to efficiencies that can be obtained in CE and CEC modes. The system also allowed the use of step flow gradient, which has further enhanced the operational flexibility of this capillary LC method.

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