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Separation and identification of etodolac and its urinary phase I metabolites using capillary electrochromatography and on-line capillary electrochromatography–electrospray ionisation mass spectrometry coupling

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Abstract

Capillary high-performance liquid chromatography (capillary HPLC), pressure-assisted capillary electrochromatography (pCEC) and capillary electrochromatography (CEC) were performed in the same capillary packed with 5 μm octadecylsilica (C_{18}) as stationary phase. These three separation modes were compared from the viewpoint of peak efficiency and separation selectivity in order to critically evaluate the advantages which CEC may offer compared to capillary HPLC for the solution of practical biomedical problems. The separation of the non-steroidal anti-inflammatory drug etodolac (ET, 1) and its phase I metabolites, 6-hydroxy etodolac (6-OH-ET, 2), 7-hydroxy etodolac (7-OH-ET, 3) and 8-(1′-hydroxyethyl) etodolac (8-OH-ET, 4) was selected as an example. Baseline separation of all compounds was achieved in different modes and conditions. The effect of pure electrophoretic separation mechanism on the overall separation selectivity observed in CEC has been shown. A high electroosmotic flow (EOF) was observed in C_{18} packed capillary even at pH 2.5 in various buffers. Furthermore, these separations were coupled on-line with electrospray ionisation mass spectrometry (ESI-MS) and the parent drug and its metabolites were identified in urine. For the coupling of CEC with ESI-MS a laboratory-made electrophoretic device was used in order to overcome some technical disadvantages of commercial instrumentation.

Keywords: Electrochromatography; Mass spectrometry; Etodolac

1. Introduction

Capillary electrochromatography (CEC) is one of the most rapidly developing microanalytical separation techniques at present [1–3]. In recent years, with the developments in column technology and appearance of several commercial capillary electrophoretic (CE) systems also adapted for use in the CEC mode, this technique has gained considerable attention. CEC at present has acquired the status of a hybrid technique of CE and high-performance liquid chromatography (HPLC) combining the best of both

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methods. In spite of rapid development the potential of CEC has still to be explored. For this, on the one hand, it is essential to get a better understanding of fundamental aspects of the EOF generation in the capillaries packed with different materials in various buffers, development of diverse modes of CEC, and the combination of electrophoretic and chromatographic separation mechanisms. To these belong, for example, separations in nonaqueous CEC [4–6], separations of charged analytes based on a combination of both electrophoretic and chromatographic mechanisms, on-line coupling of CEC with mass spectrometers (MS) [7–9], etc. The development of these techniques requires a better understanding of migration, peak dispersion and separation mechanisms in CEC. Some problems arise due to the shortcomings of commercial instrumentation. For example, an on-line coupling of CEC with MS using commercial instruments requires a column length of typically 100 cm [10,11]. This restricts the field strength and consequently, prolongs the analysis time and reduces efficiency. On the other hand, just a few studies have been published on the application of CEC for real biomedical problems [8,12].

In this study the separation of the non-steroidal anti-inflammatory drug etodolac (ET, 1) and its three hydroxylated phase I metabolites, 6-hydroxy etodolac (6-OH-ET, 2), 7-hydroxy etodolac (7-OH-ET, 3) and 8-(1′-hydroxyethyl) etodolac (8-OH-ET, 4) was performed in three different separation modes: capillary HPLC, pressure-assisted CEC (pCEC) and CEC. The same experimental set-up was used in all three modes. In addition, the separation system was optimized based on the recently proposed module [8] to simplify on-line coupling to electrospray ionisation MS (ESI-MS). This module allows not only to use a separation capillary of nearly any desired length but also a continuous replenishment of the buffer.

2. Experimental

2.1. Chemicals and reagents

Racemic etodolac (ET) and its phase I metabolites, 6-hydroxy etodolac (6-OH-ET), 7-hydroxy etodolac (7-OH-ET) and 8-(1′-hydroxyethyl) etodolac (8-OH-ET) (Fig. 1) were gifts from Wyeth (Münster, Germany). Acetic acid, acetonitrile, ammonium formate, ammonium acetate, citric acid, disodium hydrogen phosphate, formic acid, methanol, potassium dihydrogen phosphate, sodium hydroxide, thiourea were from different commercial sources and used without further purification. LiChrospher® 100 RP-18 (5 μm) used for the preparation of packed capillaries, was purchased from Merck (Darmstadt, Germany).

2.2. Preparation of capillary columns

Fused-silica capillaries of 100 μm I.D. from Polymicro Technologies (Phoenix, AZ, USA) were used. The inlet-end of the capillary was connected to an HPLC-precolumn (50×4.6 mm) which served as reservoir for the slurry of the packing material in methanol. A commercially available HPLC column frit was connected to the outlet end of the capillary in order to retain the packing material. The slurry of the packing material was ultrasonicated in a water-bath (15 min) and transferred into the reservoir. The system was closed tightly, a pressure of 400 bar was applied using a Knauer pneumatic pump (Knauer, Berlin, Germany) and maintained for 1 h. After complete reduction of the residual pressure (2–3 h), bidistilled water was pumped through the packed bed with a pressure of 200 bar for 30 min. The outlet and inlet frits were sintered by local heating of the packed bed for ≈15 s using a heating coil (700–800°C). The packed capillaries prepared according to this technique were used for capillary HPLC, pCEC and CEC separations.

2.3. Capillary HPLC, pCEC and CEC

Separations in capillary HPLC, pCEC and CEC were performed using two different experimental set-ups. Initially, the separation in all three modes were carried out using commercially available capillary electrophoresis equipment, HP^{3D} (Hewlett-Packard, Waldbronn, Germany). The total length of the capillary was 33 cm and 24.5 cm were packed with the stationary phase. The maximum pressure for capillary HPLC separations performed using HP^{3D} system was only 12 bar. The mobile phases/buffers and other separation conditions are described in the legends to the figures.

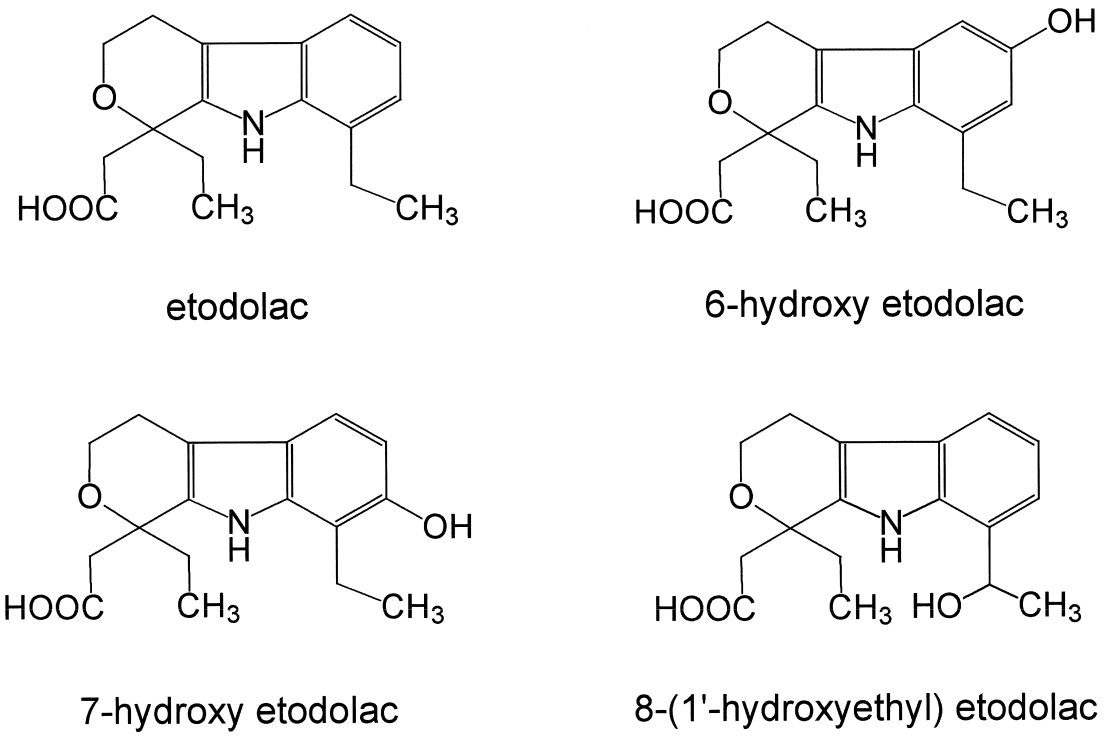

Fig. 1. Structure of etodolac (ET, 1) and its metabolites 6-hydroxy etodolac (6-OH-ET, 2), 7-hydroxy etodolac (7-OH-ET, 3) and 8-(1′-hydroxyethyl) etodolac (8-OH-ET, 4).

The injection of the sample was performed either by pressure or electrokinetically. UV detection was performed on-capillary at 220 nm immediately behind the outlet frit or through the frit.

Another experimental set-up was a laboratory-made CEC sample interface consisting of a stainless steel tee piece and a restrictor in analogy to that described recently by Taylor and Teale (Fig. 2) [8]. The stream of mobile phase generated using a commercial HPLC pump flows coaxially passing the capillary column inlet through a PEEK lead tube and leaves the tee piece through a restriction capillary. Voltage up to 27 kV was applied to the metal tee piece using a Grom capillary electrophoresis system 100 high voltage supply (Grom, Harrenberg, Germany) and the outlet of the capillary was grounded. The sample was introduced into the mobile phase stream using the HPLC autosampler and further loaded onto the separation capillary either by pressure or electrokinetically.

2.4. On-line coupling with ESI-MS

An LCQ ion trap mass spectrometer (Finnigan, Branford, CT, USA) equipped with an electrospray interface was used for the on-line coupling. As the sheath liquid served the buffer, which was also used for the separation. It was delivered at a flow-rate of 3 μl/min using a syringe pump.

2.5. Samples

ET, 6-OH-ET, 7-OH-ET and 8-OH-ET were dissolved in methanol–water (50:50, v/v) at a concentration of 75 μg/ml.

2.6. Urine samples

Blank urine samples were spiked with 300 μg/ml ET, 6-OH-ET, 7-OH-ET and 8-OH-ET and then extracted with the same volume of ethyl acetate. The

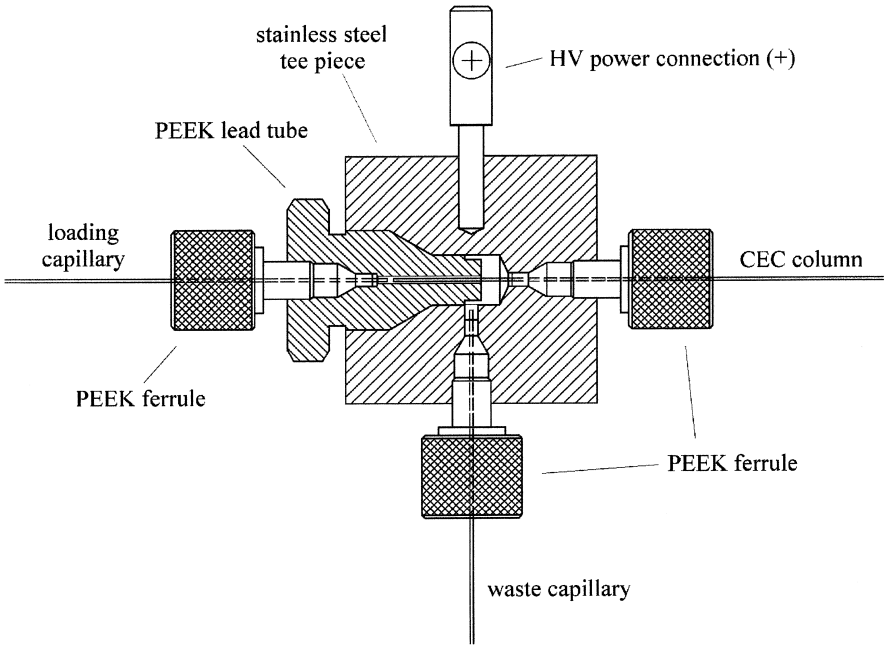

Fig. 2. CEC sample interface and experimental set-up.

organic solvent was evaporated and the residue reconstituted in the original volume of buffer. This solution was injected into the separation capillary as a sample.

3. Results and discussion

3.1. Separations with UV detection

In separations using UV detection, three separation modes, capillary HPLC, pCEC and CEC were compared. The separations in all three modes were performed in the same experimental set-up. This allowed evaluation of the effect of switching from pressure-driven to electrically-driven migration mechanisms on the separation parameters. Although not the same analysis time was adjusted for these separations some preliminary comparisons are possible to make between these techniques. In confirmation to our previous results [12] somewhat higher peak efficiency can be observed in the capillary columns compared to common size HPLC columns (Fig. 3). This effect is not significantly dependent on the migration mechanism, i.e. either the analytes are driven by pressure, or electrokinetically (Fig. 4).

One of the shortcomings of most commercial instruments which are also recommended for CEC studies is the upper limit of the high-pressure. This is 12 bar in the HP^{3D} CE equipment and therefore it was difficult to maintain an analysis time shorter than 30 min in the HPLC mode. Shorter analysis times were achieved in the CEC and pCEC modes (Fig. 4b and c). However, the plate numbers were similar in all three modes and varied between 80 000 and 135 000 per meter.

An example for creating a separation window by combining the chromatographic and the electrophoretic separation mechanism is shown in Fig. 5. The baseline separation of thiourea (TU) (nonretained component) and ET was hardly possible in a pure HPLC run. This can be explained by the low affinity of significantly deprotonated analyte towards the reversed-phase (C_{18}) material. This means that ET is weakly retained on the stationary phase at pH 6 when in addition, the mobile phase contains high amount (90%) of acetonitrile. With application of increasing voltage the separation of these two components increased continuously and at 10 kV a

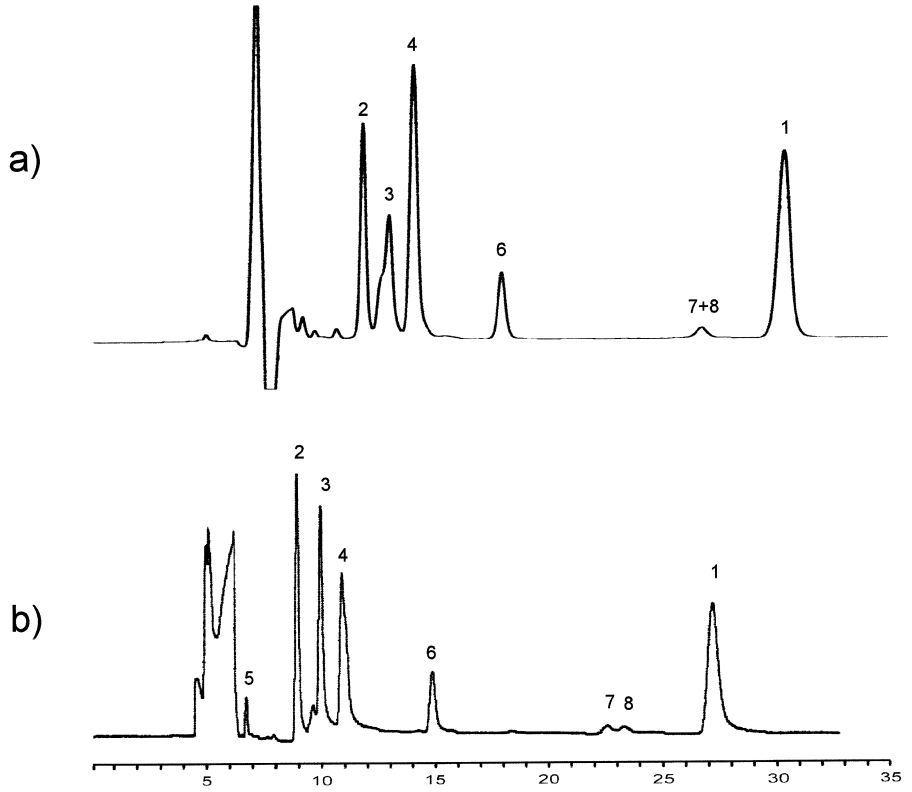

Fig. 3. Separation of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) in HPLC with common size columns (a) and packed capillary columns (b). Columns: (a) stainless steel (250×4 mm) packed with LiChrospher 100 RP-18, 5 μm; (b) fused-silica capillary (250×0.1 mm) packed with LiChrospher 100 RP-18, 5 μm; mobile phase: 10 m*M* ammonium formate pH 3.0–acetonitrile (50:50, v/v); detection: UV 222 nm (5=impurity of 3; 6=impurity of 2; 7/8=impurities of 4).

separation window of almost 3 min was obtained between TU and ET. This effect is achieved by simultaneous creation of the increasing EOF, which shortens the migration time of the non-retained compound (TU), and the oppositely directed electrophoretic mobility of the partially negatively charged ET. Similar effect has been reported recently by Apffel et al. [13].

The effect of the concentration of ammonium acetate in the buffer containing 70% acetonitrile was minor on the separation and migration times. However, the amount of acetonitrile in the separation buffer strongly affected the analysis time and peak efficiency (Fig. 6). These effects were also observed in HPLC with common size columns. The effect was stronger in CEC compared to HPLC. The increasing EOF together with the increase of eluting strength of the buffer seems to be responsible for this effect.

Reproducibility of the migration times was acceptable in CEC for performing a preliminary method validation (Table 1).

Beside the aforementioned high-pressure limit it is very difficult to run commercial CEC instruments in a gradient elution mode because of the closed buffer vials. In addition, they are not ideal for coupling CEC to a mass spectrometer, because generally very long distances have to be bridged. Therefore, a laboratory-made CEC sample interface (Fig. 2) [8] replacing the inlet vial was used in this study. This relatively simple device allows the following: (1) easy replenishment of the buffer (2) application of relatively high pressure (3) use of separation capillaries of any desired length and (4) performing the separations in a gradient mode. These advantages considerably simplify the use of a CEC–MS coupling.

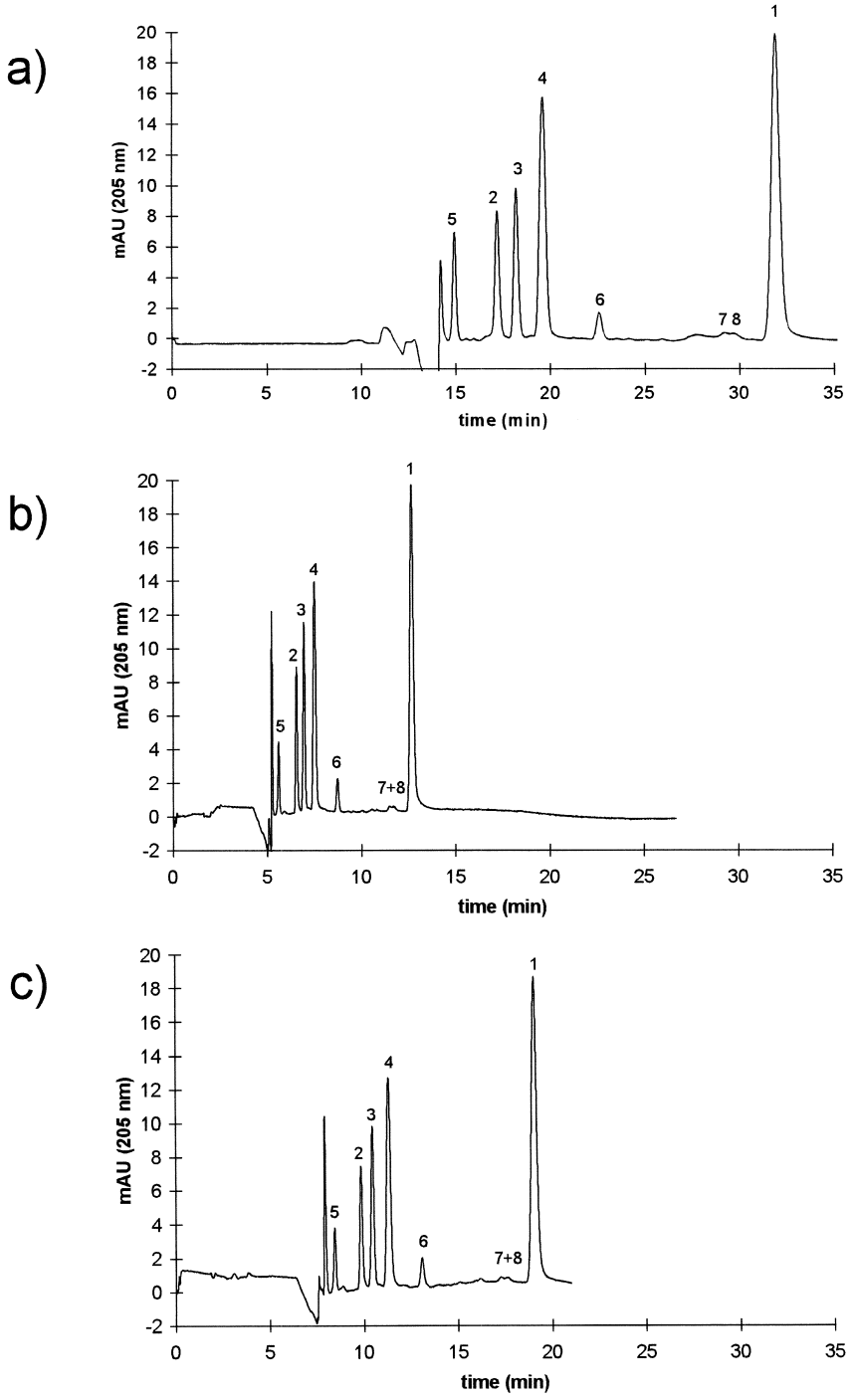

Fig. 4. Separation of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) (a) in capillary HPLC, (b) in pCEC and (c) in CEC mode. Instrument: HP^{3D}; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 75 m*M* ammonium formate pH 2.5–acetonitrile (40:60, v/v); separation conditions: (a) separation by pressure 12 bar; (b) separation by pressure 12 bar+voltage 27 kV; (c) separation by voltage 27 kV (inlet/outlet vial 12 bar); injection: by pressure 12 bar; detection: on-column at 254 nm (5=impurity of 3; 6=impurity of 2; 7/8=impurities of 4).

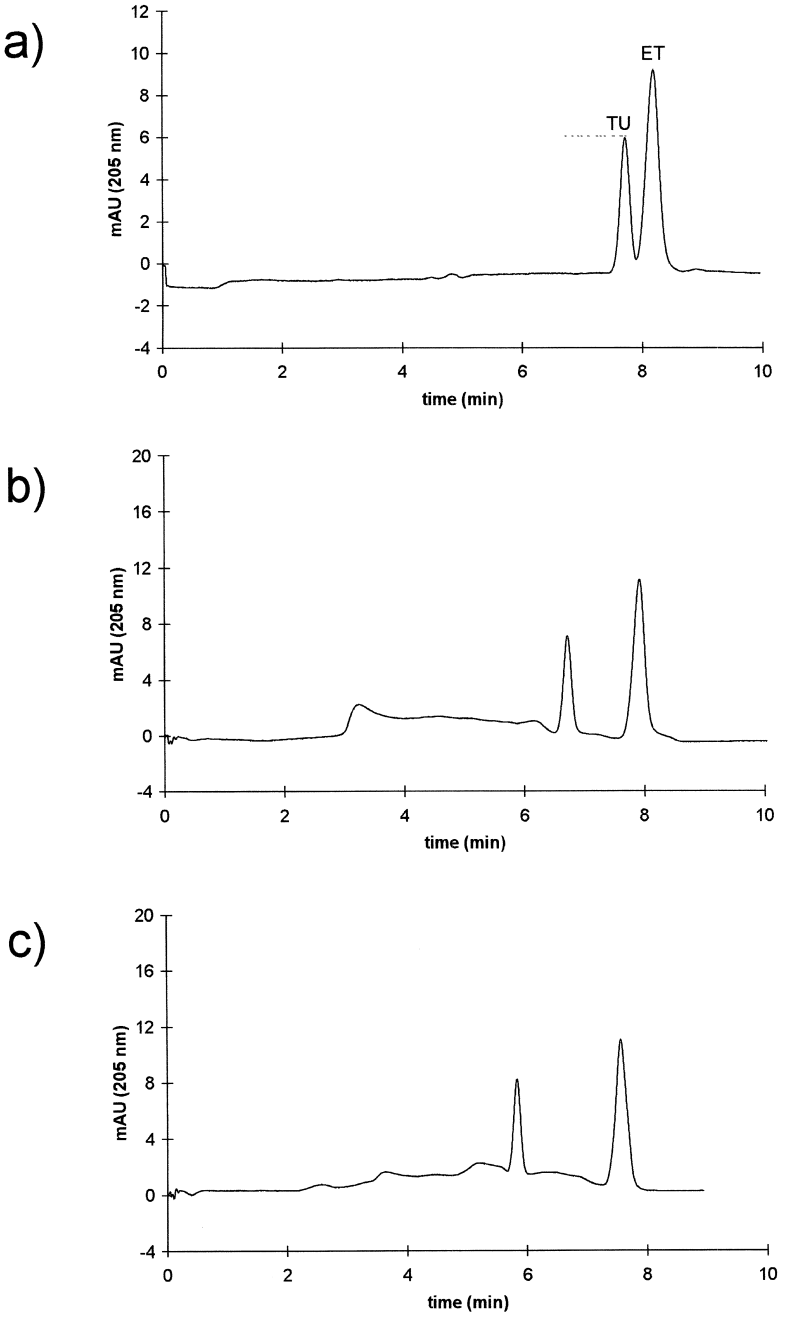

Fig. 5. Separation of thiourea (TU) and ET (a) by pressure 12 bar and by additional application of voltage (b) 2 kV, (c) 4 kV, (d) 7 kV and (e) 10 kV. Instrument: HP^{3D}; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 5 m*M* ammonium acetate pH 6–acetonitrile (10:90).

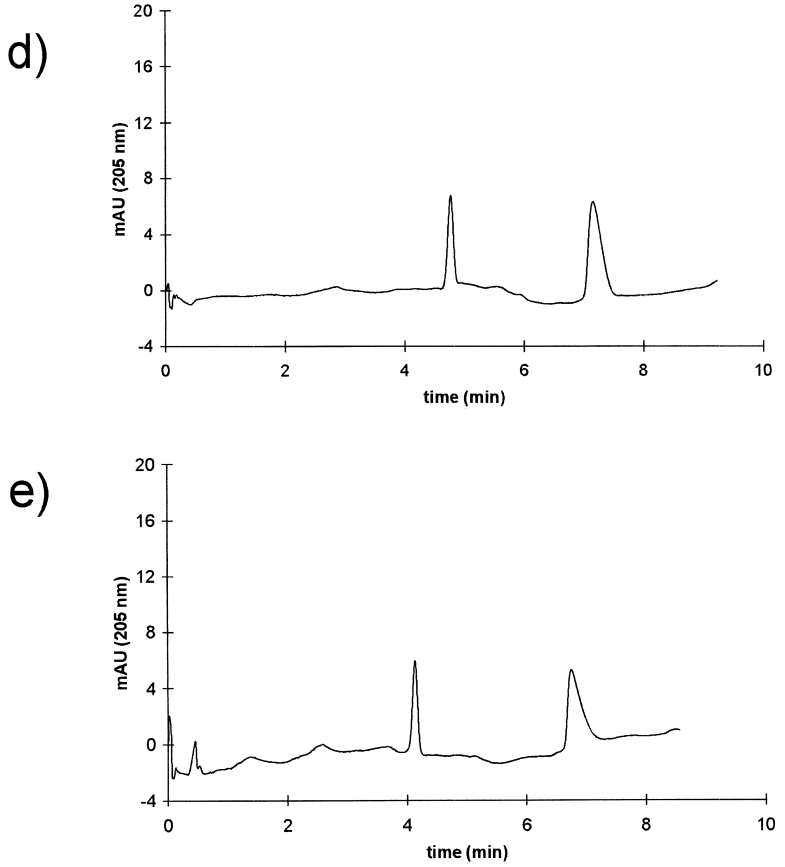

Fig. 5. (*continued*).

Table 1
Intra-day repeatability of the migration times and peak areas in CEC separation of 6-OH-ET, 7-OH-ET, 8-OH-ET and ET[a]

	Intra-day repeatability												
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Mean	SD	SD %
Migration time													
6-OH-ET	6.146	6.157	6.147	6.155	6.138	6.177	6.158	6.188	6.174	6.199	6.1639	0.01979	0.321068
7-OH-ET	6.524	6.536	6.525	6.535	6.518	6.559	6.540	6.571	6.558	6.583	6.5449	0.02176	0.33245
8-OH-ET	7.068	7.064	7.069	7.079	7.064	7.108	7.088	7.122	7.105	7.135	7.092	0.02429	0.342539
ET	11.891	11.908	11.883	11.912	11.911	11.989	11.952	12.004	12.003	12.048	11.9501	0.05730	0.479521
EOF	5.033	5.041	5.020	5.035	5.029	5.055	5.045	5.048	5.050	5.065	5.0421	0.01329	0.263687
Peak area													
6-OH-ET	24.609	22.755	16.022	20.015	21.544	24.661	23.326	22.101	26.092	25.959	22.7084	3.04614	13.41416
7-OH-ET	17.493	16.086	11.147	13.791	15.141	17.093	16.214	15.224	18.135	17.753	15.8077	2.12157	13.42114
8-OH-ET	27.659	25.491	17.819	22.158	23.641	27.225	25.422	24.501	28.658	28.668	25.1242	3.34765	13.32439
ET	32.116	29.015	20.532	24.588	26.474	30.220	28.199	27.981	32.233	32.234	28.3592	3.75997	13.25837

[a] Instrument: HP[3D]; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 75 m*M* ammonium formate pH 2.5–acetonitrile (40:60); voltage: 27 kV (inlet/outlet vial 12 bar).

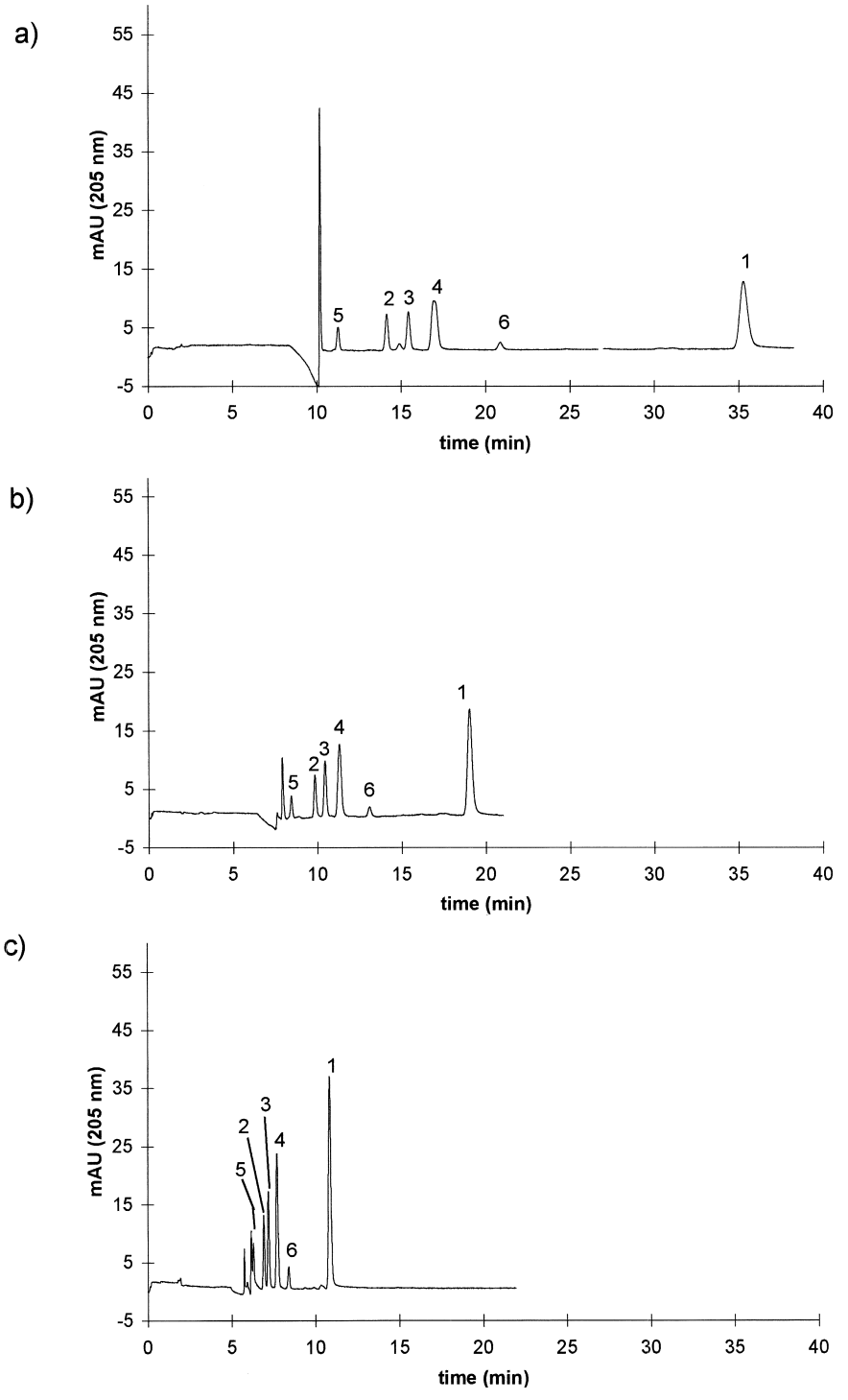

Fig. 6. Separation of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) in CEC mode with various amounts of acetonitrile: (a) 50% (b) 60% (c) 70% and (d) 80%. Instrument: HP[3D]; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 75 m*M* ammonium formate pH 2.5–acetonitrile; voltage: 27 kV (inlet/outlet vial 12 bar) (5=impurity of 3; 6=impurity of 2).

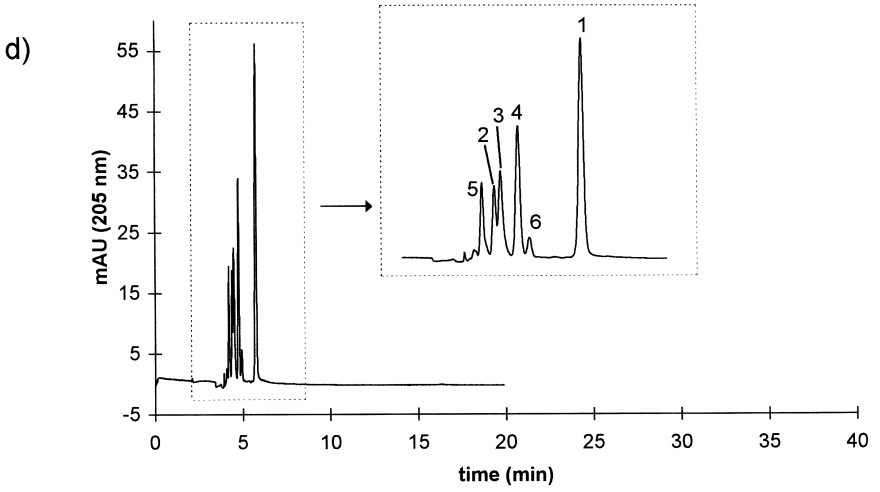

Fig. 6. (*continued*).

In this experimental set-up the effects of the type of buffer and pH on the EOF and the separation of ET and its phase I metabolites were studied. Intuitively, with the capillaries packed with C_{18} material it may be favourable to work in the pH range 6–7 where the EOF is expected to be higher. However, for the analytes which may be ionized in this pH range some complications may occur. At first, the affinity of ionized analytes will be lower to C_{18} material compared to uncharged species and this may impair the separation. In addition, the electrophoretic and chromatographic separation mechanisms will overlap in this pH range. Thus, the effect of pH of the mobile phase on the separation of ET and its metabolites was studied in both, capillary-LC and pCEC. CEC without pressure assistance was difficult to use in the desired pH range because the self-electrophoretic mobility of anionic analytes was directed in opposite direction to the EOF and in a certain pH range the analysis time became impractically long.

As shown in Fig. 7 the effect of the applied electric field is dramatic at pH>4.5. The separation which fails in this pH range in pure chromatographic mode appears again with applying voltage. This indicates that the sample components are resolved at this pH primarily electrophoretically, and the chromatographic retention plays a minor role in separation. The analysis time was shortened with applying the electric field in whole pH ranges studied. This means that the generated EOF was always higher than the self-electrophoretic mobility of the analytes. Surprisingly, the elution order was the same for all analytes in both chromatographic and electrophoretic runs. Here, the opposite could be expected.

In the pH range <4.0 the effect of the applied voltage was only a shortening of the analysis time. The separation itself which seems to be determined with chromatographic retention in this pH range was less affected with the applied field. Although already mentioned in several previous studies [14,15] the significant EOF in the low pH range in C_{18} silica gel packed capillaries seemed somewhat unusual. Therefore, four different buffers (formate, acetate, citrate, phosphate) were tested at pH 3.0. No significant difference has been observed between them either from the viewpoint of the EOF generation or the separation efficiency (Fig. 8). These buffers were applied at different concentrations in order to arrange a comparable current and analysis time with all of them.

3.2. On-line CEC–ESI-MS coupling

The on-line CEC–ESI-MS coupling was performed using the CEC sample interface shown in Fig. 2. Peak efficiencies were slightly lower in these separations (Fig. 9). However, the coupling to the

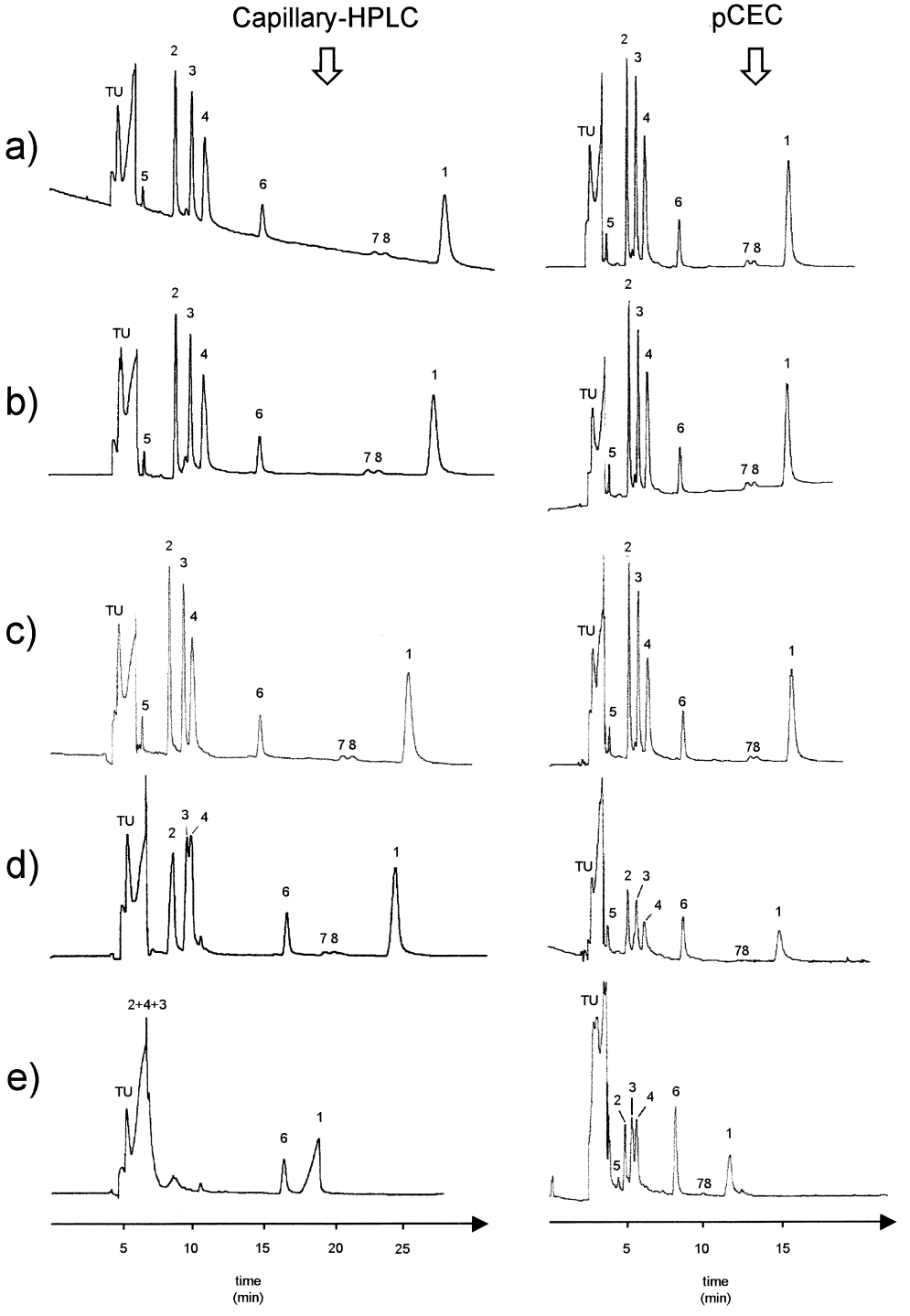

Fig. 7. Separation of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) in capillary HPLC and pCEC modes with acetonitrile–ammonium formate at different pH: (a) pH 2.8 (b) pH 3.0 (c) pH 4.0 (d) pH 4.5 and (e) pH 5.0. Instrument: laboratory-made CEC sample interface in combination with Grom CE-1000 system; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 10 m*M* ammonium formate–acetonitrile (50:50); voltage: 27 kV; injection: in HPLC mode by pressure; in pCEC mode by pressure/electrokinetically; detection: on-column at 220 nm. Applied pressure in HPLC and pCEC separations was 60 bar (5=impurity of 3; 6=impurity of 2; 7/8=impurities of 4).

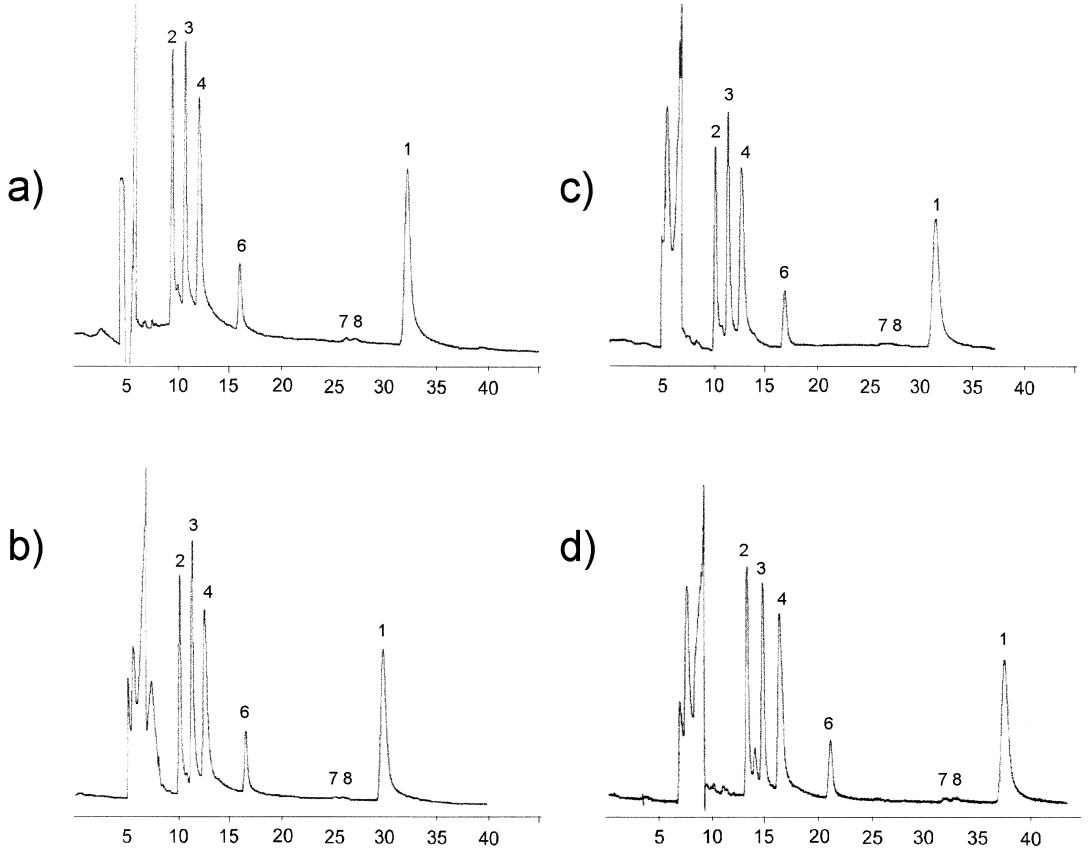

Fig. 8. Separation of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) in CEC mode in various buffers: (a) 10 m*M* formate pH 3.0–acetonitrile (50:50) (b) 5 m*M* phosphate pH 3.0–acetonitrile (50:50) (c) 50 m*M* acetate pH 3.0–acetonitrile (50:50) and (d) 20 m*M* citrate pH 3.0–acetonitrile (50:50). Instrument: laboratory-made CEC sample interface interface in combination with Grom CE-1000 system; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); voltage: 27 kV; injection: electrokinetically; detection: on-column at 220 nm (6=impurity of 2; 7/8=impurities of 4).

MS does not seem to be responsible for this effect, because the same peak efficiency was also observed when using this CEC sample interface with on-capillary UV detection. The chromatogram resulting from the total-ion-current (TIC) in full scan mode (Fig. 10a) and the selected mass-tracks (Fig. 10b and c) of the urine extract spiked with ET, 6-OH-ET, 7-OH-ET and 8-OH-ET are depicted in Fig. 10. Full scan mass spectra of each peak are shown in Fig. 10d–h. From these data it is obvious that on-line CEC–ESI-MS coupling can be used for a selective detection, identification and peak purity testing when analysing complex biomedical samples.

4. Conclusions

As shown in this study capillary HPLC, pCEC and CEC represent attractive alternatives to HPLC separations using common size columns for biomedical studies. Somewhat higher peak efficiency observed in capillary HPLC compared to common-size columns could not further be improved significantly by switching from pressure-driven to electrically-driven migration mechanism. In the capillaries packed with C_{18} material the EOF even at pH 2.5 was sufficient for driving the uncharged analytes through the packed bed. Commercially available CE instruments

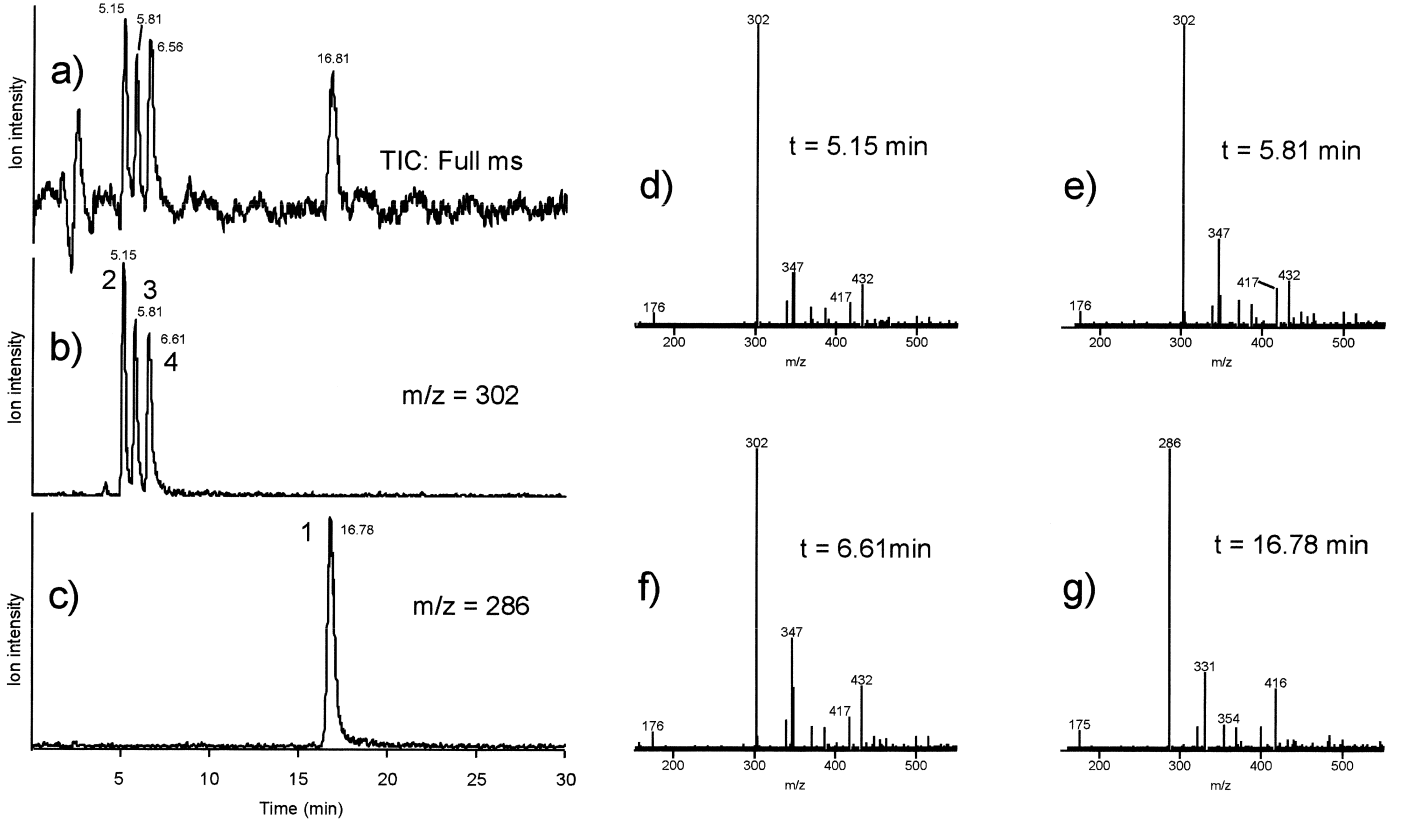

Fig. 9. CEC separation and on-line ESI-MS detection of ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) using laboratory-made CEC sample interface. (a) Chromatogram total-ion-current (TIC), full scan mode (b) selected mass track $m/z=302$ (c) selected mass track $m/z=286$ (d) full scan mass spectrum of the peak at 5.15 min (e) full scan mass spectrum of the peak at 5.81 min (f) full scan mass spectrum of the peak at 6.61 min and (g) full scan mass spectrum of the peak at 16.78 min. Instrument: laboratory-made CEC sample interface in combination with Grom CE-1000 system; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 10 m*M* ammonium formate pH 3.0–acetonitrile (50:50); voltage: 20 kV; injection: electrokinetically; detection: ESI-MS. ESI-MS conditions: polarity: negative; source voltage: 3.5 kV; sheath gas flow: 20 arbitrary units; sheath liquid: separation buffer; sheath liquid flow: 3.0 μl/min.

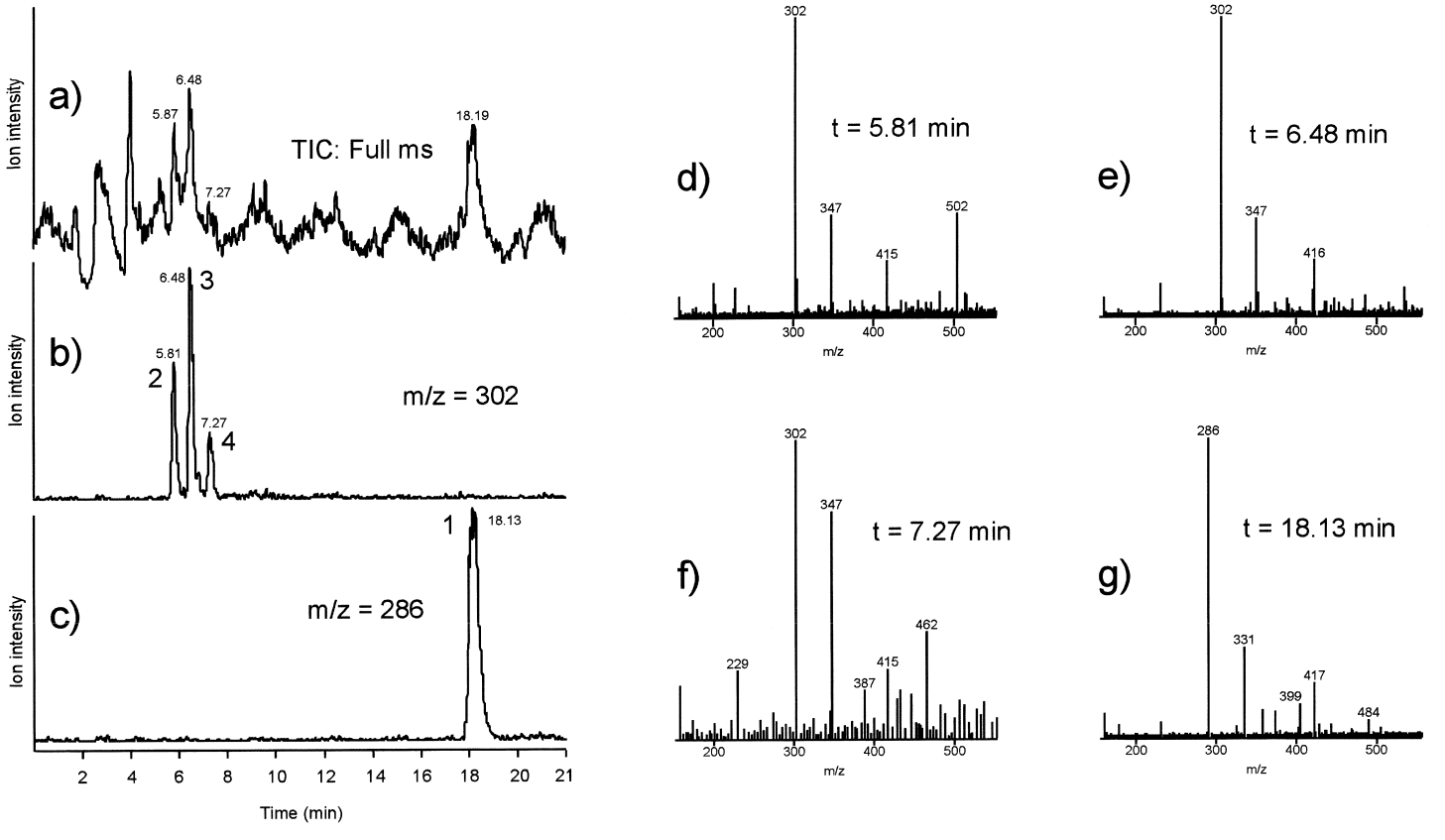

Fig. 10. CEC–ESI-MS analysis of a blank urine extract spiked with ET (1), 6-OH-ET (2), 7-OH-ET (3) and 8-OH-ET (4) (300 μg/ml) a) chromatogram total-ion-current (TIC), full scan mode; b) selected mass track $m/z=302$; c) selected mass track $m/z=286$; d) full scan mass spectrum of the peak at 5.81 min; e) full scan mass spectrum of the peak at 6.48 min; f) full scan mass spectrum of the peak at 7.27 min; g) full scan mass spectrum of the peak at 18.13 min. Instrument: laboratory-made CEC sample interface in combination with Grom CE-1000 system; capillary: fused-silica 100 μm I.D., 23 cm packed (LiChrospher 100 RP-18, 5 μm); buffer: 10 m*M* ammonium formate pH 3.0–acetonitrile 50:50; voltage: 20 kV; injection: electrokinetically; detection: ESI-MS. ESI-MS conditions: polarity: negative; source voltage: 3.5 kV; sheath gas flow: 20 arbitrary units; sheath liquid: separation buffer; sheath liquid flow: 3.0 μl/min.

which are partly adapted also for CEC applications are not ideally suited for this purpose; a relatively simple laboratory-made CEC sample interface where the closed inlet buffer vial is replaced by a metal tee piece allows to replenish the inlet buffer continuously during the run, application of nearly any desired pressure and length of the capillary and in principle, working in a gradient elution mode. The on-line coupling of CEC with MS becomes much easier using this device. The CEC–ESI-MS coupling is feasible and seems to be a very useful technique for biomedical studies of complex samples.

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