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Capillary electrochromatography of steroids
Increased sensitivity by on-line concentration and comparison with high-performance liquid chromatography

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Abstract

A reversed-phase HPLC method previously developed for the analysis of progesterone and its major metabolites has been transferred successfully to a capillary electrochromatography (CEC) system. Procedures for fabricating packed capillaries and the modifications made to the capillary electropherograph which allow operation in the CEC mode without pressurisation are described. The dependence of electroosmotic flow on electric field strength, pH and organic modifier content is discussed. Direct comparison with HPLC shows that CEC provides useful gains in efficiency and speed of analysis and requires vastly reduced amounts of both chromatographic phases and material for analysis. On-line concentration is described which allows the lower sensitivity of CEC to be offset by injecting analytes from a non-eluting solution. Examination of steroids in plasma demonstrates that the superior separation by CEC is maintained in a complex biological matrix. © 1998 Elsevier Science B.V.

Keywords: Electrochromatography; Sensitivity enhancement; Steroids; Progesterone

1. Introduction

In capillary electrochromatography (CEC), separations resulting from the combined effects of electrophoresis and partition chromatography occur within fused-silica capillaries (50–100 μm I.D., up to 100 cm in length) which are packed with silica gel-based stationary phases similar to those used in high-performance liquid chromatography (HPLC) [1,2]. Mobile phase is driven through the capillary column by electroosmosis [3]. It has been shown that CEC is capable of producing separations of considerably higher efficiency than HPLC [4], partly because the electroosmotic flow (EOF) has a flatter profile than pressure-driven flow, but also because injection, separation and detection are all accomplished within the same capillary thus eliminating extra-column effects. Detection can be performed within the packed section, increasing efficiency still further but at the expense of sensitivity [5,6]. CEC, in common with other capillary separations, shares the further advantages of high mass sensitivity and negligible sample and mobile phase consumption. However, for many real applications, particularly those in biological systems, concentration sensitivity is the factor which limits the usefulness of the capillary techniques [7].

For neutral compounds which are not amenable to separation by capillary zone electrophoresis (CZE) there may be a need for an alternative technique to HPLC which provides greater resolution, or which can be justified on the basis of reduced running costs

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and generation of hazardous waste solvents. Micellar electrokinetic capillary electrochromatography (MEKC) was developed as such an alternative but has several drawbacks which limit its usefulness. These include a restricted choice of 'pseudo-stationary phase', a limited separation window for neutral analytes which elute with a velocity between that of the mobile phase and the micelles, and incompatibility with mass spectrometric detection because of the high concentrations of surfactants used.

Most of the published data for CEC have been limited to separations of model test mixtures at relatively high concentrations, and, although the results are impressive with efficiencies of up to 300 000 theoretical plates per metre being claimed for a 3 μm diameter stationary phase [4], it remains to be seen whether such performance is achievable for analytes in more complex matrices (e.g. biological fluids) at realistic concentrations and also whether the conditions required for optimised separations by CEC are comparable with those for HPLC.

The aims of the present investigation are to study the basic requirements and operating characteristics of a CEC system, to try to overcome the inherently low concentration sensitivity—a limiting factor for many bioanalyses, to directly compare the performance of CEC and HPLC for the same separation, and to assess whether the performance of CEC is maintained for analytes in a complex biological matrix.

We used the separation of progesterone and its major metabolites, a very demanding analysis previously performed on microbore HPLC [8], to compare the performance of CEC and standard analytical HPLC systems. Steroids were examined in plasma in order to assess the effect of a complex biological matrix on the separation and to determine whether it is possible to approach the levels of detection necessary to measure endogenous levels of steroids.

2. Experimental

2.1. Chemicals and reagents

Steroids supplied by Sigma (Poole, UK) were dissolved in methanol (1 mg/ml) from which working solutions were prepared by dilution. Stock solutions (2 mg/ml) were also prepared for thiourea (Fisons, Loughborough, UK) in water and for propyl-*p*-hydroxybenzoate (BDH, Poole, UK) in acetonitrile–20 mM Tris·HCl (pH 8) (80:20, v/v) and diluted as required. Acetonitrile and methanol were obtained from Rathburn (Walkerburn, UK). Water was glass-distilled and further purified using a Milli-Q system (Millipore, Watford, UK).

2.2. Capillary electrochromatography

CEC experiments were performed using an Isco Model 3850 capillary electropherograph (supplied by Crawford Scientific, Strathaven, UK) with the following modifications made to the capillary inlet arrangement. The inlet end of the capillary was routed into a free-standing inlet buffer beaker through a beaker cover (Isco part no. 60-3144-058) instead of via the rotary manifold (which was loosened and moved out of the way, keeping the rotary knob in the RUN position). Electrical connection from the electrode post to the inlet beaker cover was made using an extra Ground Lead (Isco part no. 60-3144-057). Injections were made by substituting the inlet buffer beaker for another, either containing the sample solution (5–8 ml required) or a polystyrene insert (Fig. 1) which allowed injections to be made from micro-vials (20–100 μl required), then applying a run voltage (up to 30 kV) for the

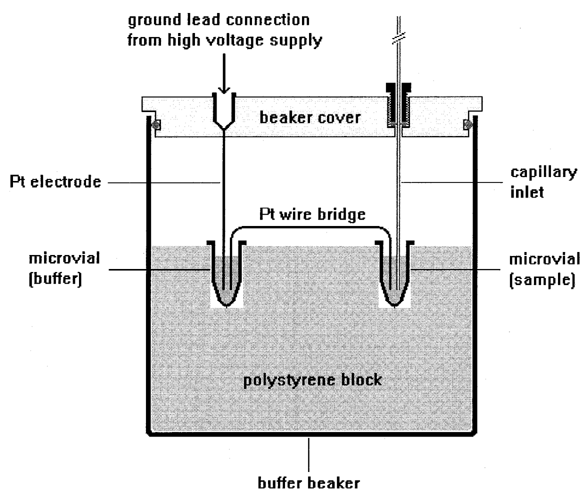

Fig. 1. Modified inlet buffer beaker for injection from small volume samples.

duration required. Detection was performed on-column in an unpacked section of the capillary adjacent to the outlet frit at a wavelength of 240 nm and rise time setting of 0.8 s. Chromatograms were recorded on a BBC Goertz Metrawatt Model SE120 chart recorder with manual measurement of peak height and width.

The mobile phase was generally prepared by mixing previously filtered and degassed acetonitrile, methanol and 20 mM Tris·HCl (pH 8) in the required ratio and then further degassing for 1–2 min by combined application of vacuum and ultrasound. Use of the low conductance buffer Tris, combined with rigorous degassing of the mobile phase, allowed CEC runs to be performed at up to 30 kV without the need for pressurisation of the system to suppress bubble formation [2].

2.3. Fabrication of packed capillary columns

Lengths (50 cm) of flexible fused-silica capillary (100 μm I.D.×363 μm O.D.; Polymicro Technologies, supplied by Composite Metal Services, Hallow, UK) were packed with 3 μm ODS Hypersil (HETP, Macclesfield, UK) by a similar procedure to that described by Boughtflower et al. [9,10]. Capillaries were packed with slurry (0.1 g stationary phase in 1.5 ml acetonitrile) at 10 000 p.s.i. using a syringe pump (Isco Model 100DM) and acetonitrile as the pumping solvent (1 p.s.i.=6894.76 Pa). A 0.5 μm pore size stainless-steel SSI Precolumn Filter (Supelco, Poole, UK) was used in place of an end frit to retain the stationary phase particles during packing. The former is more reliable, convenient and speeds up the packing procedure considerably.

When packing was complete (generally within 10 min), the capillary was depressurised in a controlled manner. The syringe pump is ideal for this purpose since it has the facility to perform pressure gradients. After removal of the reservoir, the capillary was purged with water at 10 000 p.s.i. for at least 15 min, the pressure was reduced to 6000 p.s.i. and an outlet frit created 20 cm from the in-line filter. Frit formation was achieved by sintering a short (1–2 mm) section of the packing material using an electrically heated filament. This consisted of a 5 cm length of Nichrome wire (N80, 0.32 mm diameter, a kind gift from Graham Hall, Kanthal, Stoke-on-Trent, UK) with a single winding in which the capillary was a close fit, connected to a B30/20 Stabilised Power Supply (Farnell Instruments, Leeds, UK) in series with a multimeter for monitoring of the current. It was possible to produce strong frits using a current as low as 2.4 A for 60 s without damaging the polyimide coating. This is a major advantage over methods which cause the coating to be burned away during sintering, rendering the capillary extremely fragile.

Acetonitrile–water (80:20, v/v) was pumped overnight at 10 000 p.s.i. in the reverse direction to consolidate the packed bed and remove excess stationary phase downstream of the outlet frit. After purging again with water, an inlet frit was created as described above at a suitable distance from the pump connection to give the desired packed length (15–40 cm can be accommodated in the modified Isco capillary electropherograph).

Column fabrication was completed by forming a detection window immediately downstream of the outlet frit, using the electrically heated filament at higher current (3.5–4 A) to burn away the polyimide coating, and trimming the capillary inlet end to within 2 mm of the inlet frit. The column was purged with rigorously degassed mobile phase at up to 4000 p.s.i. for at least 1 h before installation in the CEC system. Prior to performing analyses, newly installed columns were equilibrated at up to 5 kV for several hours.

2.4. High-performance liquid chromatography

The HPLC system comprised a Waters Model 510 Pump (Millipore), a Rheodyne Model 7125 valve fitted with a 20 μl loop, a stainless-steel column (20 cm×4.6 mm I.D.) slurry-packed with 3 μm ODS Hypersil (as used in the CEC columns), and a Jasco UV-975 Intelligent UV–Vis Detector (Jasco UK, Great Dunmore, UK) connected to a chart recorder for manual peak measurement as before. Mobile phase was prepared as for CEC.

2.5. Sample preparation for plasma analyses

Bond-Elut C_{18} solid-phase extraction cartridges (100 mg, 1 ml; Varian, Harbor City, CA, USA) were conditioned with 1 ml methanol then 1 ml water. The

plasma sample (0.5 ml) was applied, and the cartridge washed with 2 ml water followed by 2 ml 20% methanol. Steroids were eluted with 2×0.5 ml volumes of methanol. The extract was reduced to dryness in a stream of nitrogen and redissolved in 50 μl of acetonitrile–methanol–20 mM Tris (pH 8) (22.5:22.5:55, v/v/v) giving a 10-fold increase in concentration.

3. Results and discussion

3.1. Effect of voltage on electroosmotic flow (EOF) and efficiency

CEC analyses of a test mixture containing thiourea (25 μg/ml) and propyl-*p*-hydroxybenzoate (50 μg/ml) in the mobile phase of acetonitrile–20 mM Tris·HCl (pH 8) (80:20, v/v) were performed using a capillary with a packed length of 25 cm and total length of 45 cm. Injections were made for 5 s at 10 kV and the run voltage was varied from 1–30 kV. The variation in linear mobile phase velocity, estimated from the retention time of thiourea (a virtually unretained marker), with the applied electric field strength is shown in Fig. 2. The relationship between linear velocity and electric field strength showed a degree of non-linearity at voltages above 3 kV. This seems unlikely to be caused by heat generation within the capillary since the current did not exceed 5 μA even at maximum voltage.

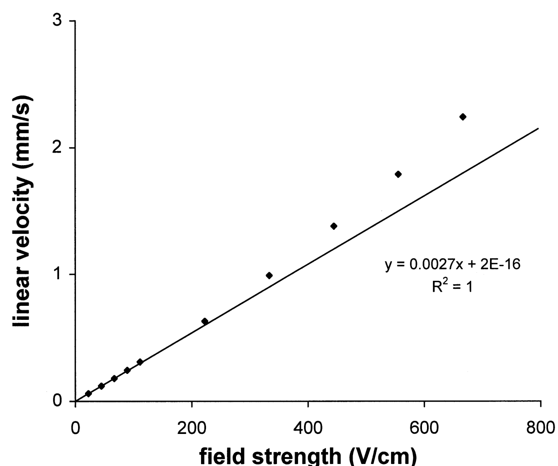

Fig. 2. Plot of mobile phase linear velocity vs. applied electric field strength. Conditions: 25 (45) cm×100 μm I.D. capillary packed with 3 μm ODS Hypersil, run in acetonitrile–20 mM Tris·HCl (pH 8) (80:20, v/v). The linear regression line is shown for the data points at 22, 44 and 66 V/cm (applied voltage 1, 2 and 3 kV, respectively), with predicted extensions covering the complete voltage range (0–30 kV).

Fig. 3 shows the corresponding *H*–*u* curve for the retained component propyl-*p*-hydroxybenzoate. It was evident that the high efficiency was not compromised at linear mobile phase velocities of at least 2 mm/s. Flat *H*–*u* curves have also been reported elsewhere for electrically driven packed capillaries, in contrast to pressure-driven columns for which the plate height increased steeply with increasing linear velocity [11]. This may be a particularly useful advantage of CEC over HPLC, and suggests that very fast separations may be possible by CEC, if packed capillaries can be developed which are capable of generating the high EOF necessary at reasonable electric field strengths. Some studies have already looked at the characteristics of the stationary phase which affect the magnitude of the EOF [4,12] with conflicting results, but manufacturers (e.g. Hypersil) are already beginning to produce stationary phases with enhanced EOF for CEC applications.

3.2. Effect of pH and organic modifier content on EOF

Fig. 4 shows the effect of varying the pH of the

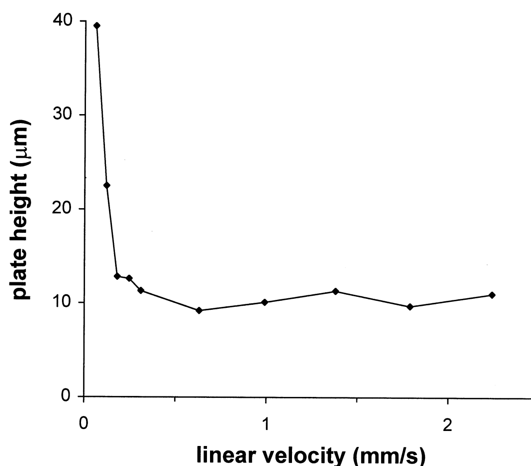

Fig. 3. Plot of height equivalent to a theoretical plate vs. mobile phase linear velocity for the retained test compound propyl-*p*-hydroxybenzoate. Conditions as for Fig. 2.

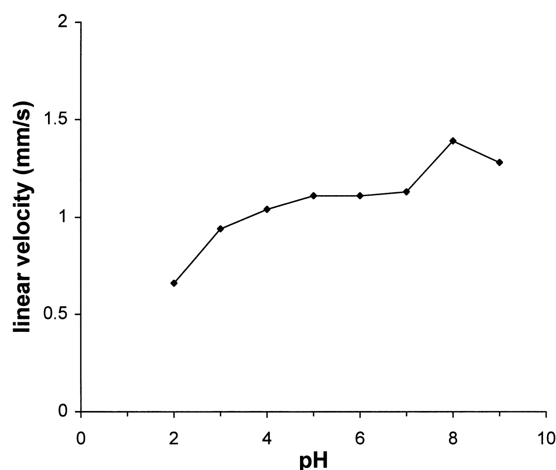

Fig. 4. Plot of linear mobile phase velocity vs. pH. Conditions as for Fig. 2, with fixed run voltage of 20 kV.

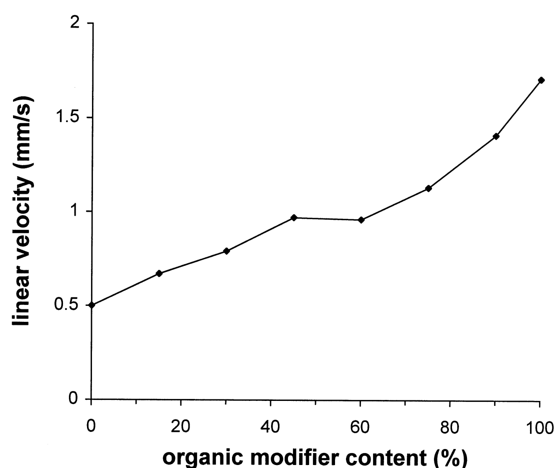

Fig. 5. Plot of linear mobile phase velocity vs. organic modifier content. Conditions as for Fig. 2, except that the organic modifier was acetonitrile–methanol (1:1, v/v) and the run voltage was 25 kV.

aqueous component (20 mM Tris, pH adjusted by the addition of HCl) on the linear mobile phase velocity, using the same conditions as described above, at a fixed run voltage of 20 kV. It should be noted that Tris has limited buffering capacity outside the range pH 7–9. The relationship between linear velocity and pH which was observed is consistent with the theory of EOF generation [1,3]. As the pH falls, the surface silanols become ionised to a lesser extent and the EOF decreases.

The effect of varying the organic modifier content on the linear mobile phase velocity is shown in Fig. 5. Linear velocity was estimated as before in a 25 cm packed (45 cm total length) capillary at a fixed run voltage of 25 kV. The mobile phase consisted of varying proportions (0–100%) of organic solvent (acetonitrile–methanol, 1:1, v/v) and water, while keeping the total electrolyte concentration at 5 mM. The observed increase in linear velocity with higher organic modifier content is consistent with the expected changes in mobile phase viscosity and dielectric constant and EOF theory. Similar results have been reported for binary mobile phase systems [4,11], although other workers have found the reverse [13,14]. These differences have yet to be resolved.

The dependence of the EOF on the mobile phase conditions is undesirable from a method development standpoint. Ideally it should be possible to vary pH and organic modifier content to manipulate the selectivity and retention of analytes independently of the flow velocity, as in HPLC. Development of stationary phases and capillary coatings which generate a constant EOF over a wide pH range would therefore be a useful advance.

3.3. Effect of injection time (zone length) on peak height and width using eluting and non-eluting solutions ('on-line concentration') in CEC

Test solutions were prepared containing the steroid testosterone (20 μg/ml) in (i) an 'eluting solution' consisting of the mobile phase, acetonitrile–methanol–20 mM Tris·HCl (pH 8) (37.5:37.5:25, v/v/v), and (ii) a 'non-eluting solution' of acetonitrile–methanol–20 mM Tris·HCl (pH 8) (22.5:22.5:55, v/v/v), and analysed on a 25(45) cm capillary at a run voltage of 25 kV. Fig. 6 shows the effect of varying the duration of injection (at a constant voltage of 25 kV), and, therefore, the volume or zone length of material introduced into the capillary, on the testosterone peak.

For the eluting (high solvent strength) solution, a 5 s injection introduced the largest volume of sample before efficiency was markedly compromised. Longer injections from the eluting solution caused the peak width to increase sharply, while the peak

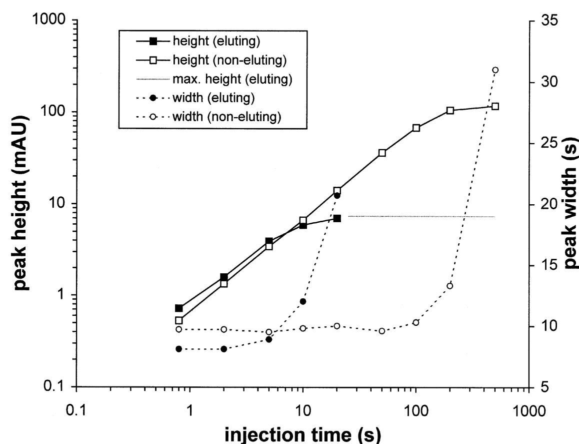

Fig. 6. Plots of peak height and width for testosterone injected from eluting (75%, v/v, organic modifier content) and non-eluting (45%, v/v, organic modifier content) solutions vs. duration of injection. Conditions as for Fig. 5, injection voltage 25 kV. The maximum peak height for the eluting solution was obtained by continuously injecting the sample and measuring the absorbance change from the baseline.

height approached a plateau which corresponded to a maximum absorbance achievable by continuous injection of the sample solution.

In contrast, it was possible to make a 100 s injection from the non-eluting (low solvent strength) sample solution without noticeable loss of efficiency or change in retention of the testosterone peak. The relationship between peak height and injection time showed good linearity up to 100 s (r^2 for the linear regression equation=0.999). Moreover, a 17-fold increase in peak height was achieved under these conditions when injecting from the non-eluting solution compared with the high solvent strength solution. Such an 'on-line concentration' effect has been used in HPLC to preconcentrate analytes by retention at the front of the column, but has only recently been described in CEC [15]. The authors reported a 10-fold preconcentration factor but did not go on to study the effect in detail.

3.4. Qualitative comparison of steroid separations by CEC and HPLC

The separation of progesterone and its metabolites 17α-hyroxyprogesterone, 20α-hyroxyprogesterone, androstenedione and testosterone plus norethindrone (internal standard) was previously optimised for microbore HPLC (10 cm×1 mm, 5 μm ODS Hypersil column) [8]. Adequate separation of all the components within 30 min could only be achieved by maximising retention and selectivity with a ternary solvent and performing the analysis at a high flow-rate (0.1 ml/min, which is equivalent to 2.1 ml/min in a standard 4.6 mm I.D. column). In order to obtain a direct comparison with CEC, the HPLC separations were performed on a standard analytical HPLC column with the same packed length and stationary phase as used in the CEC column. The operating parameters are shown in Table 1. The chromatograms which were obtained under these conditions are shown in Fig. 7A and B.

The retention times for all the components corresponded remarkably well, showing that these neutral steroids are separated essentially by partition chro-

Table 1
Operating parameters for separation of steroids by CEC and HPLC

	CEC	HPLC
Packed column dimensions	20 cm (35 cm total) × 0.1 mm I.D.	20 cm×4.6 mm I.D.
Stationary phase	3 μm ODS Hypersil	3 μm ODS Hypersil
Mobile phase	CH_3CN–CH_3OH–20 mM Tris·HCl (pH 8) (37.5:37.5:25, v/v/v)	CH_3CN–CH_3OH–20 mM Tris·HCl (pH 8) (37.5:37.5:25, v/v/v)
Linear velocity	5 cm/min (0.83 mm/s) at 15 kV	5 cm/min (0.83 mm/s)
Volume flow rate	0.3 μl/min estimated	0.6 ml/min
Injection volume	25 nl estimated (5 s at 15 kV) (~2% of column void volume)	20 μl (~0.8% of column void volume)
Detection	240 nm, range 0.02 AU	240 nm, range 1.28 AU
Sample	Thiourea, testosterone, androstenedione, 17α-hyroxyprogesterone, 20α-hyroxyprogesterone, norethindrone and progesterone, each at 20 μg/ml in mobile phase	

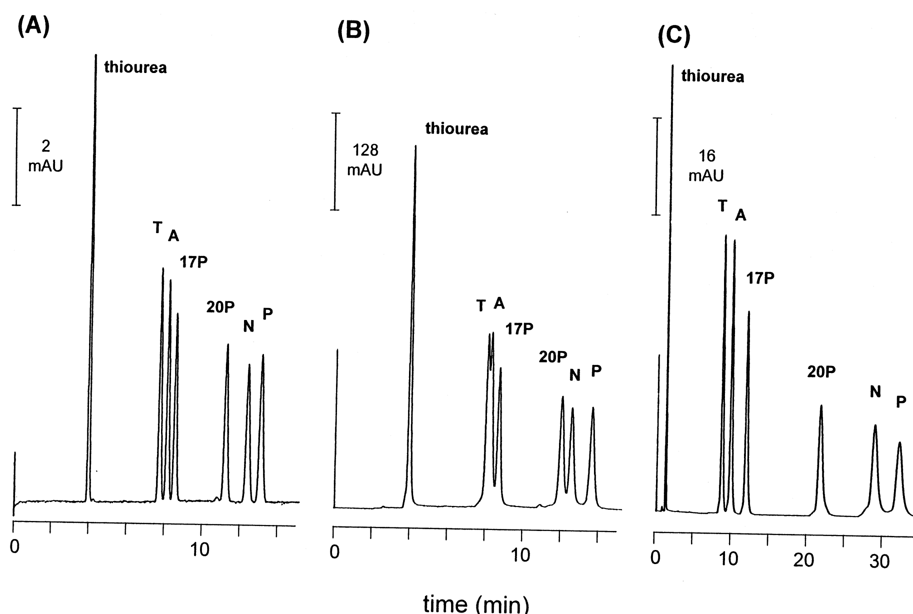

Fig. 7. Separations of progesterone [P] and its metabolites 17α-hyroxyprogesterone [17P], 20α-hyroxyprogesterone [20P], androstenedione [A] and testosterone [T] plus norethindrone [N, internal standard] and thiourea, obtained by: (A) CEC, and (B) HPLC using the conditions described in Table 1, and (C) HPLC using a mobile phase of CH_3CN–CH_3OH–20 mM Tris·HCl (pH 8) (30:20:50, v/v/v) and a flow rate of 2.1 ml/min. Each steroid was present in the test mixture at 20 μg/ml.

matography in CEC, as in HPLC. However, two major differences are apparent between the two techniques. Firstly, the higher efficiency of CEC allowed baseline resolution of all components with a total analysis time of 13 min, whereas by HPLC only progesterone was fully resolved within a similar time. Secondly, the relative sensitivity of CEC, estimated from the absorbance response for the testosterone peak, was 47-fold less than HPLC. If the difference in the proportion of sample volume as a percentage of column void volume (see Table 1) is taken into account, then CEC is found to be roughly 100-fold less sensitive than HPLC. This is explained by the shorter path length over which the absorbance measurements are taken, i.e. 0.1 mm for CEC against 10 mm for HPLC. Improvements in capillary detector design, such as Z- or U-shaped flow cells, which enable path length to be increased to at least 1 mm without dilution of the separated peaks will therefore bring the concentration sensitivity of CEC closer to that of HPLC.

An optimised separation of the steroid mixture using the standard analytical HPLC system is shown for further comparison in Fig. 7C. Conditions were as described in Table 1, except that the mobile phase was acetonitrile–methanol–20 mM Tris·HCl (pH 8) (30:20:50, v/v/v), the flow-rate was 2.1 ml/min (giving a back-pressure of 5500 p.s.i. which was close to the limit for the pump) and the absorbance range was 0.16 AU. The components were fully resolved only by using a lower solvent strength (50% organic modifier) and running at a high flow-rate, as previously observed using microbore HPLC. Even so the fastest analysis time was more than twice that which could comfortably be achieved by CEC.

3.5. Analysis of steroids in plasma by CEC with on-line concentration

Fresh frozen human plasma (obtained from the NE Scotland Blood Transfusion Service, Aberdeen, UK) was spiked with the same mixture of six steroids, each at a final concentration of 200 ng/ml. Following solid-phase extraction, the plasma extracts were dried and redissolved in non-eluting solvent to give a 10-fold preconcentration (see Section 2). Electro-

chromatograms for the plasma extracts analysed on a 25(45) cm capillary, run at 25 kV and injected for varying times at 25 kV are shown in Fig. 8.

The marked gain in sensitivity without loss of resolution produced by on-line concentration was clearly observed for injections of at least 60 s. The chromatogram illustrated in Fig. 8D shows that using the on-line concentration technique, it is possible to approach the sensitivity required to detect endogenous levels of steroids in plasma, even without the gains in sensitivity which are now available with improved detector design.

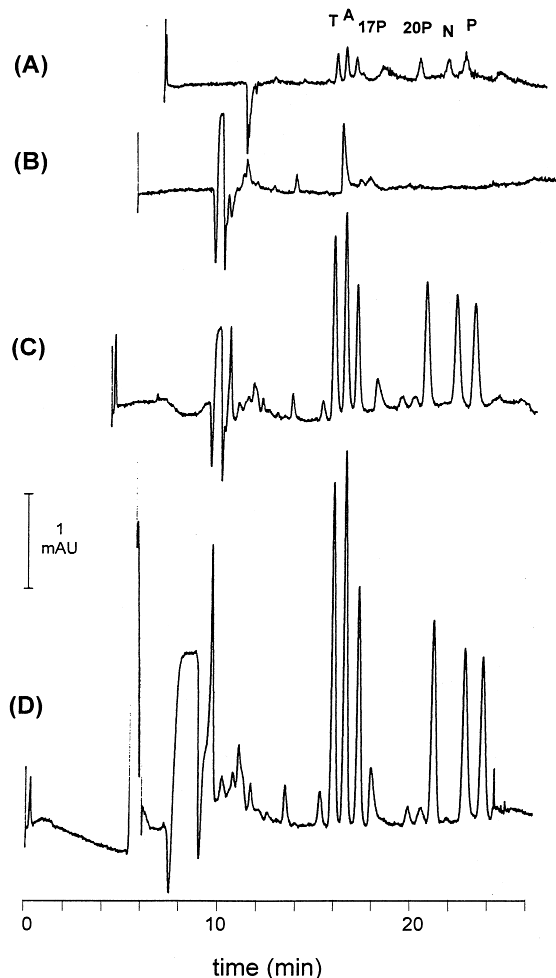

Fig. 8. Capillary electrochromatograms of plasma extracts, injected at 25 kV from non-eluting solutions for: (A) 5 s, (B) and (C) 30 s, or (D) 60 s. For chromatograms (A), (C) and (D) the original plasma was spiked with the same six steroids as in Fig. 7 at 200 ng/ml, while chromatogram (B) is from unspiked plasma. Conditions and elution order as for Fig. 7A.

As the injection time increased, the retention time of each steroid increased uniformly. This is seen in Fig. 8 as a lengthening of the initial portion of the chromatogram, while the steroid peaks can still be aligned in the same relative positions. This was not observed in the experiments described above (Section 3.3) and is, therefore, likely to be caused by factors associated with the plasma matrix.

It is interesting to note that the large solvent front which is generally found in HPLC analyses of plasma was not observed when using CEC. Chromatograms previously published for the comparable microbore HPLC analysis of steroids in plasma [8] showed a large solvent front which interfered with the early eluting testosterone and androstenedione peaks. It may be the case that in CEC, ionic matrix components, which would elute at the front of an HPLC analysis creating a very large tailing peak, are either selectively removed by the electrokinetic injection process (for anions) or accelerated through the column faster than the mobile phase (for cations) so that they no longer interfere with the analyte peaks. This may be a general advantage of CEC over HPLC when performing bioanalyses.

A report has recently been published showing the separation of three corticosteroids (hydrocortisone, dexamethasone and fluocortolone) in equine urine using a gradient CEC system [16]. The aims were not stated and it is difficult to understand the rationale behind the study since the compounds should be easily separable by conventional techniques. The authors themselves state that an existing validated LC–MS procedure is preferable for this particular application, having a much shorter analysis time. The report also showed the detection of the single steroid hydrocortisone at a level of 5 μg/ml in plasma (i.e. 25 times more concentrated than that described for testosterone in the present study) following dialysis, in which 250 μl of dialysate was consumed by the injection system and the gradient of acetonitrile served to concentrate the sample at the front of the column. From the data provided, it can be calculated that the amount of sample actually injected would be equivalent to that from a 150 s injection at 25 kV. Although a direct comparison with the results shown above and in Section 3.3 is

not possible since the CEC column dimensions are different, we would expect such a large injection to significantly reduce the separation efficiency. It was not shown whether the gradient CEC system could effect useful separations under these conditions.

4. Conclusions

We have demonstrated that a reversed-phase HPLC method, previously developed for the analysis of progesterone and its major metabolites, can be transferred successfully to CEC with minor modification. For the first time, a direct comparison of CEC and HPLC is presented which shows that the same factors which control the separation of neutral analytes in HPLC also operate in CEC. The higher efficiency which is predicted for CEC is observed practically in the improved resolution of the steroid analytes and a decreased analysis time. On-line concentration of the steroids is described, both from a simple non-eluting solution and a plasma extract, which provides up to a 17-fold increase in sensitivity. Using a combination of solid-phase extraction with sample preconcentration and the on-line concentration technique described here, it is possible, with improvements in capillary detection systems now available, to reach the levels of detection required for the analysis of endogenous steroids in plasma. These advances will open up the possibilities for using CEC to perform many other useful bioanalyses.

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