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Steroid profiles determined by capillary electrochromatography, laser-induced fluorescence detection and electrospray–mass spectrometry

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Abstract

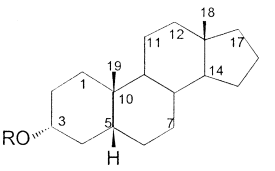
Macroporous, monolithic capillary electrochromatography (CEC) columns, featuring a hydrophobic stationary phase, have been applied to the separations of steroids with good column efficiency. Using isocratic and gradient elution runs, mixtures of neutral or conjugated steroids could be resolved. While dansylated ketosteroids were detectable through laser-induced fluorescence at attomole levels, the CEC columns coupled to electrospray–ion-trap mass spectrometry featured femtomole detection limits. © 2000 Elsevier Science B.V. All rights reserved.

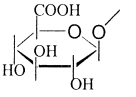
Keywords: Electrochromatography; Monolithic columns; Steroids

1. Introduction

Steroids comprise a large group of natural substances that must frequently be monitored in various biological materials. Additionally, numerous synthetic steroids have been made as therapeutic agents. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of a chromatographic procedure prior to detection/measurement. This has now been demonstrated with profiling steroids for a disease diagnosis and endocrinological research [1–4], in pharmacokinetic studies, and in identifying the abuse of anabolic steroids in sports.

A frequent complication of various steroid determinations is their biological conjugation in the forms shown below, as exemplified by a hydroxy substitution at position 3:



	R
free steroids	H–
sulfate conjugates	
glucuronide conjugates	

To begin with, most biologically important steroids can have a polar substitution at C-3, C-11 and C-17

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positions, featuring in their free forms either hydroxy or ketonic groups and, alternatively, a dihydroxyacetone chain at C-17 (originating from the biological oxidations of their common precursor, cholesterol). Aromatization of the first steroidal ring further yields the class of estrogens with the C-3 phenolic character. The formation of conjugates (sulfates and glucuronides) increases substantially the polar character of analytically studied biological steroids.

The complexity of biologically relevant mixtures demands analytical separation techniques with great resolving power and highly sensitive detection, such as capillary gas chromatography–mass spectrometry (GC–MS) [3,4]. On one hand, to overcome the natural limitations of volatility with this class of compounds, the steroids must first be freed from their conjugate forms (through enzymatic treatment or solvolysis) and doubly derivatized [1] prior to GC–MS. These steps introduce significant procedural complexities and long times for the overall analysis. On the other hand, useful information on biological conjugation is lost when resorting to the GC–MS techniques.

While analyzing steroids in a solution rather than in the gas phase would appear to be a preferable approach, this area is not without methodological complications either. Although different types of steroids are directly amenable to various modes of high-performance liquid chromatography (HPLC), the limitations of its resolving power have been apparent. Additionally, a lack of chromophores in most naturally occurring steroids is a significant problem for HPLC detection. In efforts to improve both the separation efficiency and detection for the biological steroids isolated from urine and blood serum, microcolumn liquid chromatography of benzoylated hydroxysteroids with UV detection and fluorescently labeled steroids for laser-induced fluorescence (LIF) was carried out [5–7]. Enhanced separation efficiencies (up to 100 000 theoretical plates/m) were achieved, albeit at the expense of excessive analysis times.

This report describes our first uses of capillary electrochromatography (CEC) for a significant improvement in steroid multicomponent analysis. Because the separation principles of CEC are largely chromatographic, selectivity-based separations can be developed to address different analytical problems in the area. Simultaneously, the unique flow profile associated with the electroosmotic flow (EOF) [8] is the key ingredient in achieving the favorable mass transfer and high column efficiencies needed for the analysis of complex mixtures. However, to make CEC attractive for steroid analysis, suitable means of detection must be developed as well. The 'more traditional' approaches may involve precolumn derivatization, as shown below with the use of laser-induced fluorescence (LIF) for dansylated ketosteroids. A more universal approach to detection is represented here with the coupling of our CEC columns [9,10] to an ion-trap mass spectrometer.

In a departure from the commonly pursued CEC columns (packed with siliceous particles), we have developed highly efficient macroporous matrices, which are based on a polyacrylic gel technology [9,10]. Certain advantages of these 'monolithic' CEC columns stem from the easy attachment of the matrix to the wall, eliminating the need for retaining frits and the problems with gas bubble formation. With no subsequent need for electrode reservoir pressurization, these columns appear very suitable for electrospray ionization (ESI) mass spectrometry (MS). Additionally, the basic technology of these macroporous monolithic columns permits a relatively easy modification of various functionalities on the separation gel, and provides the needed stabilities over a wide range of pH and organic solvents.

While the CEC flow-rates are compatible with a miniaturized ESI source [11], the use of MS as an on-line ancillary tool also becomes attractive for component identification in complex biological mixtures. As shown here, and in a companion study on bile acids [10], the ion-trap mass spectrometer can serve as an on-line universal detector while providing key structural information on the analytes. For most applications, CEC–MS offers a good alternative to micellar electrokinetic chromatography–MS and capillary electrophoresis–MS because of the interferences due to micelles and salts, respectively, with these methods [12]. As shown elsewhere [10] with bile acids, the choice of CEC column can also be optimized with respect to the use of a tandem (MS–MS) mode.

In this report, we show the use of a hydrophobic CEC column [10] for the separations of various

groups of steroids, including both the neutral steroids and their conjugates. Ketosteroids were derivatized with dansylhydrazine and detected through an LIF detector. A complex mixture isolated from human pregnancy urine was separated with the aid of gradient elution. For future identification of steroids in complex mixtures, the CEC system was coupled to an ESI ion-trap mass spectrometer.

2. Experimental

2.1. Chemicals

Acrylamide, *N*,*N*′-methylene-bis-acrylamide, and dansylhydrazine were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), 3-methacryloxypropyltrimethoxysilane (Bind-Silane), polyethylene glycol (PEG, MW 10 000) and all steroid standards (for structures, see Fig. 1) were received from Sigma (St. Louis, MO, USA). Vinylsulfonic acid (sodium salt, 25%, v/v), lauryl acrylate (C_{12}, 90%), and *N*-methylformamide (99%) were the products of Aldrich (Milwaukee, WI, USA).

2.2. Column preparation

Fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA), with 100 μm I.D. (360 μm O.D. for LIF detection and 160 μm O.D. for MS detection), was used to make the CEC columns. The columns were prepared according to the procedures described elsewhere [9,10].

2.2.1. Activation of capillary tubing

The inner wall of the capillary tubing was treated with 1 *M* sodium hydroxide, 0.1 *M* hydrochloric acid (both statically, for 2×15 min), and rinsed finally with distilled water for 30 min. A 50% (v/v) Bind-Silane (bifunctional reagent) solution in acetone was then introduced into the capillary and left inside for 2×20 min. Finally, the capillary was rinsed with acetone and water.

2.2.2. Polymerization procedure

(A) Linear polymer. Since off-gel fluorescence detection (see below) had to be used to increase the sensitivity of detection, the column was filled with the separation gel only up to one-half of the optical window. Consequently, the detection was achieved in free solution. The gel-free part of the capillary was coated with linear polyacrylamide [13] to limit band broadening and to decrease the risk of bubble formation. The gel-free, polyacrylamide-coated part of the column was prepared by the following procedure: 20 mg of acrylamide (4%, g/mL) was dissolved in 0.5 mL of 50 m*M* NaH_2PO_4–50 m*M*

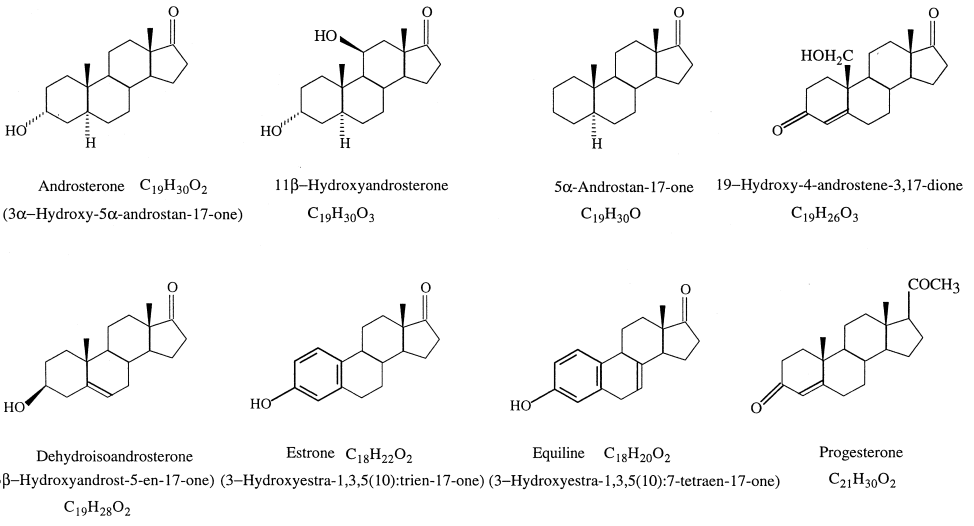


Fig. 1. Structures of standard steroids.

Na_2HPO_4 buffer (pH 7.2). The solution was then deaerated. Free radical polymerization started after 5 μL of 10% TEMED was added, followed by adding 5 μL of 10% ammonium persulfate. The monomer solution was then moved through the gel-free part of the capillary that had been freshly activated. The polymerization proceeded overnight at room temperature. (B) CEC column containing 5% T, 60% C, 3% (w/v) PEG, 10% vinylsulfonic acid and 15% C_{12} (lauryl acrylate). Here, *T*% refers to the total monomer concentration (g/mL), and *C*% refers to the total cross-linker concentration (g/g) [14]. Thirty milligrams of acrylamide, 60 mg of bis-acrylamide, 24.8 μL of vinylsulfonic acid, 60 mg of polyethylene glycol and 24.5 μL lauryl acrylate were dissolved in 1.85 mL of *N*-methylformamide–100 μL of 100 m*M* Tris–150 m*M* boric acid (pH 8.2). During the polymerization step, 4 μL of 100% TEMED and 10 μL of 40% ammonium persulfate were added to 0.5 mL of the above monomer solution. The polymerization proceeded overnight at room temperature. The capillary column was then washed with an appropriate buffer, and pressurized by applying compressed nitrogen gas at 100–200 p.s.i. The column was then conditioned by gradually applying an electrical force in an appropriate operating buffer. The well-conditioned columns were stored in the buffer before use.

2.3. CEC–LIF system

A home-built capillary electrophoresis (CE) system with laser-induced fluorescence (LIF) detection, as described previously [15], was used in this study. Briefly, the separation column was enclosed in a Plexiglas box with an interlock safety system. The fluorescence measurements were performed with a helium–cadmium laser (Model 56X, Omnichrome, Chino, CA, USA) as light source (5 mW output at 325 nm). The incident laser beam was aligned to its optimum by adjusting the position of the detection window on the column and the collecting optics. Fluorescence emission (>495 nm) was collected through a 0.6 mm fiber-optic placed at right angles to the incident laser beam. The signals isolated by a long-pass and low-fluorescence filter (Oriel, Stradford, CT, USA), removing the wavelengths below 494 nm, were monitored with a R928 photomultiplier tube (Hamamatsu Photonics K.K., Iwata-Gun, Shizuoka Prefecture, Japan) and amplified with a Model 128A lock-in amplifier (EG&G Princeton Applied Research, Princeton, NJ, USA).

2.4. CEC–ESI–ion-trap MS

Fig. 2 presents a schematic diagram of the CEC–ESI–MS system employed in this study. The setup consisted of a 60 kV high-voltage power supply (Spellman, Plainview, NY, USA) connected to a platinum electrode in a vial containing the mobile phase. A nanospray interface similar to the design described by Ji et al. [11] was constructed in-house. A nanospray needle (tapered needles with approximately 10 μm orifice were used) was prepared from fused silica (250 μm I.D., 360 μm O.D.) using a laser-based micropipette puller (Sutter, Novato, CA, USA). The separation capillary (100 μm I.D., 165 μm O.D.) had to be inserted into the fused-silica needle so that the column outlet was placed well inside the tapered portion of the needle. A sheath liquid was used to form the circuit between the column outlet and the needle, which was maintained at +1–2 kV applied through a 30 kV high-voltage power supply (Spellman). Another role of the sheath

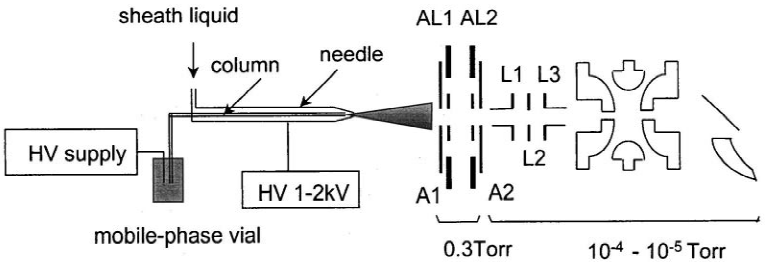


Fig. 2. Schematic diagram of the CEC–ESI–MS system.

liquid is to provide optimum pH conditions for the positive-ion electrospray ionization. A solution of acetonitrile–water–formic acid (50:49:1, v/v/v) was used as a sheath liquid in our positive-ion mode. The sheath liquid was introduced at a flow-rate of 0.5 μL/min through a micro-flow syringe pump (Model μLC-50, Isco, Inc., Lincoln, NE, USA). A 100 MΩ resistor was used to float the needle above the ground potential and also to provide a path for the separation current to ground.

The ion-trap mass spectrometer was constructed in our laboratory using an ITMS kit obtained from Finnigan (San Jose, CA, USA). This system included the ion-trap electrodes, the electronics package, and software for the data system. A vacuum system based on a design described by Van Berkel et al. [16] was constructed in-house. This system was modified and fitted with a 'home-built' electrospray ionization (ESI) source, based upon the system previously described by McLuckey and co-workers [17,18]. The ion catcher mass spectrometry (ICMS) software used to control the trap and acquire data was kindly supplied by Yates and Yost [19]. To facilitate ion injection and trapping in the ITMS, helium gas was admitted into the ion-trap vacuum housing and maintained at a steady pressure of 10^{-4}–10^{-5} Torr. Under the normal operation mode, the mass range of our ITMS was 650 amu. When detecting ions larger than 650 amu, resonance ejection mode [20] was used to extend the mass range.

2.5. Gradient CEC system

The CEC system incorporated with a mobile-phase gradient system is shown in Fig. 3. The gradient system consists of two pumps, a 200 μL micro-mixer and a specially designed anode buffer reservoir. A program controller to obtain the desired flow-rate and mobile-phase composition controlled the two pumps. Two different solvents delivered by the two pumps were then mixed in the micro-mixer with a magnetic stirrer. A mobile phase was generated at a flow-rate of 0.2 mL/min and carried into a specially designed anode buffer reservoir through a channel. The inlet of the capillary column was placed in the channel. The diameter of the channel was designed to be small (about 1 mm in diameter) so that mobile phase can flow through the capillary column at a controlled gradient without dilution. The reservoir was built from a piece of Teflon block, containing both an inlet and an outlet to keep a constant buffer flow through the reservoir.

2.6. Preparation of standards, buffer solutions and samples

2.6.1. Steroid stock solutions

Steroid standards were dissolved separately in methanol to make each stock solution (1–2 mg/mL) and stored at −4°C until use.

2.6.2. Labeling reaction for fluorescence detection

The steroids to be analyzed were derivatized for fluorescence detection using a labeling reaction (Scheme 1) described previously by Kawasaki and co-workers [21]. In particular, an aliquot of the standard or sample was evaporated to dryness before the labeling reaction. The steroid residues were mixed with 0.2 mL of 5 mg/mL trichloroacetic acid in ethanol and 50 μL of 2 mg/mL dansylhydrazine in ethanol. The reaction was carried out at 60°C in a water bath for 20 min and, finally, the solution was evaporated to dryness under a stream of nitrogen. The labeled sample redissolved in 100 μL of methanol and its aliquot was injected into the CEC system. The derivatives, excited with a He–Cd laser at 325 nm, showed the maximum emission wavelength at 530 nm.

2.6.3. Sample preparation

To extract steroids from urine, a modified method has been developed, based on the previously reported procedures [22–25]. A 5 mL volume of urine from a pregnant woman was applied onto a Sep-Pak C_{18} (Waters, Milford, MA, USA) cartridge and washed successively with 5 mL of water to remove the salts. Conjugated and neutral steroids retained on the cartridge were eluted with 8 mL of methanol, and the collected fraction was then evaporated to dryness under a stream of nitrogen gas. To hydrolyze the conjugated steroids into neutral compounds, 5 mL of 1 *M* acetate buffer (pH 5) and 10 mg of β-glucuronidase (Sigma) were added to the residue in

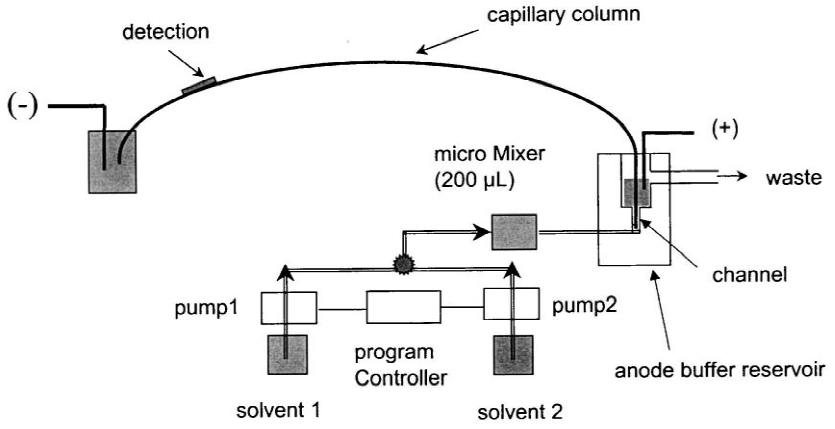


Fig. 3. Schematic diagram of the gradient CEC system.

the vessel. The contents in the vessel were then incubated overnight at 37°C. After hydrolysis, a Sep-Pak C_{18} cartridge was used to purify the liberated neutral steroids, followed by successive washing with 5 mL water. The neutral steroids were further purified through an anion-exchange column (7 mm × 50 mm) with QAE Sephadex resin (Sigma). This step was necessary to remove the naturally fluorescent pigments, which seem to have been negatively charged, interfering with the fluorescence detection of labeled steroids. The final sample was a non-fluorescent and colorless solution. The eluent was then evaporated to dryness and redissolved in 100 μL of methanol for final analysis.

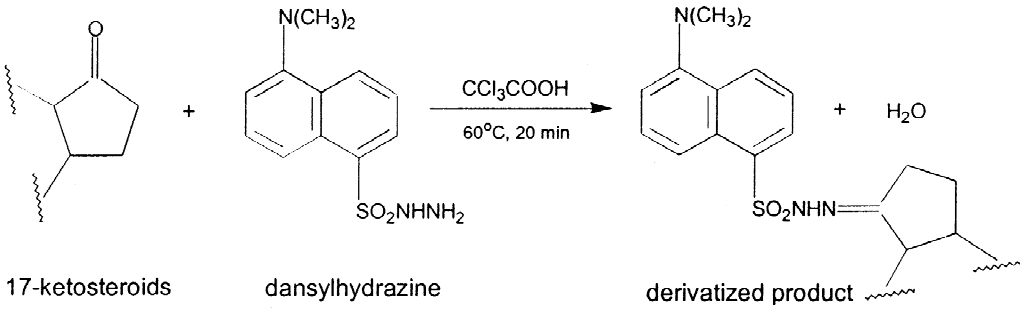
2.6.4. CEC running buffer

Ammonium formate buffer (pH 3.0, 240 m*M*) was prepared by titrating 1% (v/v) formic acid with ammonium hydroxide until the pH reached 3.0. This buffer solution was then further diluted 20 times with acetonitrile and water to make the running buffer.

3. Results and discussion

3.1. Separation of standard mixtures

The analytical problems associated with steroid analysis may vary in demands on the separation capabilities and detection sensitivity, ranging from the separation of synthetic preparations and their impurities to a highly sophisticated recording of the profiles of hormone metabolites extracted from biological materials. The primary goal of this communication has been to demonstrate the potential of CEC and its detection techniques for a range of such



Scheme 1. Derivatization scheme for labeling ketosteroids with dansylhydrazine.

analyses. This necessarily includes the ability to separate, quantify and identify free steroids, steroidal conjugates (i.e., sulfates and glucuronides) and their derivatives with chromophoric tags. Depending on their different functional groups, various steroids may vary in hydrophobicity, so that a CEC hydrophobic phase [9,10] appears appropriate for use. Moreover, buffer pH manipulation can be beneficial in facilitating the resolution of some steroid conjugates.

First, a standard mixture of six neutral ketosteroids was derivatized with dansylhydrazine prior to their separation by a hydrophobic CEC column and their detection by LIF (helium–cadmium laser at 325 nm). 11β-Hydroxyandrosterone, dehydroisoandrosterone, equiline, androsterone, 19-hydroxy-4-androstene-3,17-dione and 5′-androstan-17-one, all introduced onto the column from 5–20 μg/ml solutions (~6 nL injections, ~37 nL/min at a field strength of 100 V/cm), were driven by electroosmotic flow to separate according to their hydrophobicities (Fig. 4). The least polar compound, a singly dansylated 5′-androstan-17-one, elutes as the last component (peak 6), while the more polar components migrate earlier. Since 19-hydroxy-4-androstene-3,17-dione has two carbonyl groups on opposite sides of the steroid molecule, it separates into two distinct entities (one is derivatized at c-3 and the other at c-17), described as peaks 5a and 5b. Dehydroisoandrosterone and equiline seem to have similar hydrophobicities, coeluting in peak 3. Their coelution was further verified through CEC–MS (see below). The unidentified peak after the labeling reagent (peak 1) is due to an impurity in dansylhydrazine. With this mixture type, the column separation efficiency corresponds to about 200 000 theoretical plates/m, which is routinely achievable.

Fig. 5 presents the separation of five standard conjugated steroids: dehydroisoandrosterone sulphate, androsterone sulphate, 11β-hydroxyandrosterone glucuronide, dehydroisoandrosterone glucuronide, and androsterone glucuronide (at 20–50 μg/ml levels). Conjugated steroids are weak acids with a pK_a of ≤3.5 [26]. Under the used mobile-

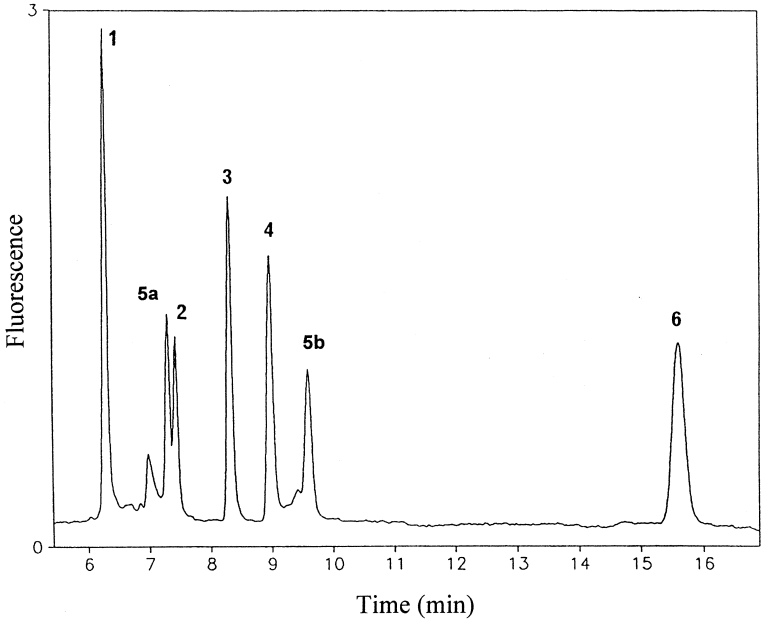


Fig. 4. Electrochromatogram of derivatized neutral steroids. Peaks: 1=labeling reagent, 2=11β-hydroxyandrosterone, 3=dehydroisoandrosterone and equiline, 4=androsterone, 5=19-hydroxy-4-androstene-3,17-dione and 6=5α-androstan-17-one, all at 5–20 μg/mL. Experimental conditions: capillary, 35 cm×100 μm (I.D.) with 360 μm (O.D.); column length, 25 cm; mobile phase, acetonitrile–water–240 m*M* ammonium formate buffer (pH 3) (55:40:5, v/v/v); field strength, 600 V/cm; current, 11 μA; injection, 100 V/cm, for 10 s.

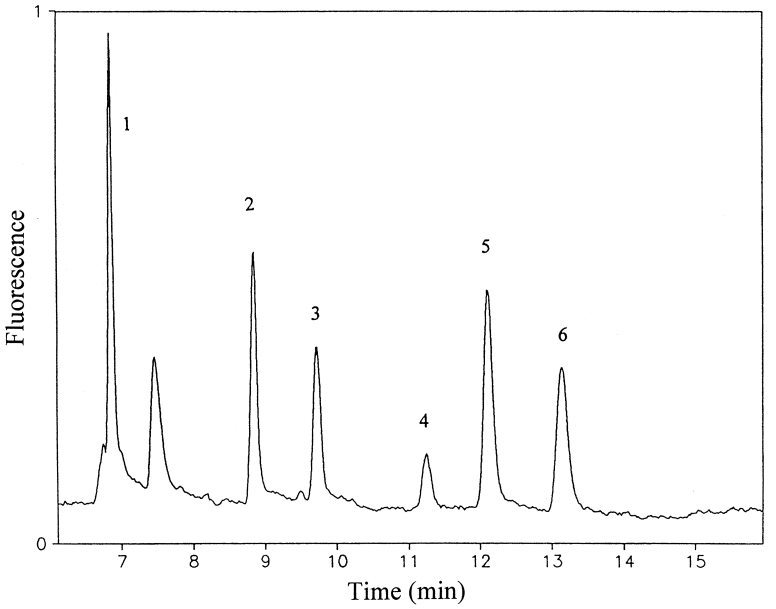


Fig. 5. Electrochromatogram of derivatized conjugated steroids. Peaks: 1=labeling reagent, 2=dehydroisoandrosterone sulphate, 3=androsterone sulphate, 4=11β-hydroxyandrosterone glucuronide, 5=dehydroisoandrosterone glucuronide and 6=androsterone glucuronide, all at 20–50 μg/mL. Experimental conditions: same as in Fig. 4.

phase composition (pH 3.0), the conjugated steroids are partially dissociated into negative ions. The negatively charged solutes appear to move slowly due to the opposing effects of electroosmosis and electrophoretic flow. Thus, in comparison with the mixture of neutral steroids, the conjugates tend to elute at longer retention times. Once again, the unidentified peak in Fig. 5 is the previously mentioned reagent impurity.

To assess the quantification potential for the dansylation–LIF procedure, calibration curves for the neutral steroids (0.05–5 μg/ml), sulphates (5–50 μg/ml), and glucuronides (1–10 μg/ml) were determined. Although the response factors for neutral and conjugated steroids were different, all tested model steroids showed good linearity (fluorescent intensity vs. concentration), indicating that they react readily with dansylhydrazine. The detection limits for neutral steroids were roughly 10 ng/ml (~100 attomoles, on column) and 500 ng/ml (~4 femtomoles, on column) for the conjugates. The assessment of detection limits was based on $S/N = 3$. However, when assessing the value of dansylated steroids in CEC–MS (see below), we found that the standard and natural mixtures contained both derivatized and underivatized compounds as distinct peaks, indicating that derivatizations proceeded in roughly 50% yields. It thus appears that the dansylation–LIF approach can be used quantitatively (maintaining reproducible derivatization conditions and standardization), as the underivatized, remaining steroids are not detectable through LIF. However, as discussed further below, the dansyl derivatives are not particularly suitable for MS identification.

3.2. Gradient elution

As with any other chromatographic method, CEC can benefit from gradient elution for optimization of separations. A number of systems with a varying degree of sophistication have recently been reported [27–29] towards this goal. In this study, a simple gradient CEC system, consisting of two pumps, a controller, and a specially designed anode buffer reservoir, was set up to demonstrate the merits of gradient elution in steroid separations. Different gradient profiles can quite easily be controlled in the

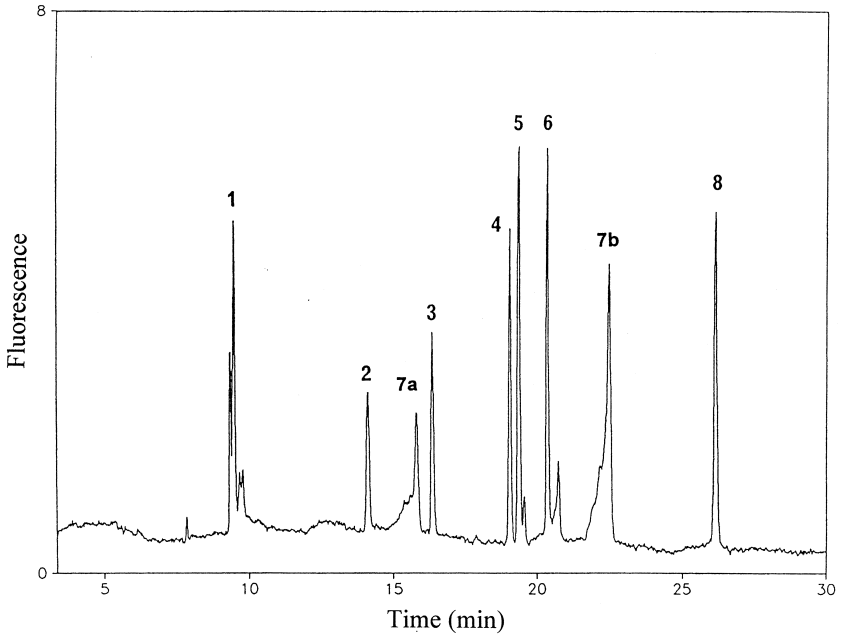


Fig. 6. Gradient electrochromatogram of derivatized neutral steroids. Peaks: 1=labeling reagent, 2=progesterone, 3=11β-hydroxyandrosterone, 4=dehydroisoandrosterone and equiline, 5=estrone, 6=androsterone, 7=19-hydroxy-4-androstene-3,17-dione and 8=5α-androstan-17-one, all at 5–20 μg/mL. Experimental conditions: gradient mobile phase, acetonitrile–water–240 m*M* ammonium formate buffer (pH 3) (35:60:5–65:30:5, v/v/v); current, 13–8 μA; the remaining conditions are the same as in Fig. 4.

system. As shown in Fig. 6 with a standard neutral steroid mixture, this procedure yields sharp and symmetrical peaks, with the notable exception of 19-hydroxy-4-androstene-3,17-dione (peaks 7a and 7b). The gradient elution thus allows a separation of components with a range of hydrophobicities in a reasonable analysis time. The mobile-phase composition of this gradient was changed from 35 to 65% acetonitrile in 15 min and kept isocratic until the last peak was eluted. These hydrophobic, monolithic CEC columns are not susceptible to irreproducible changes during gradient elution [29]. Moreover, they can tolerate an organic percentage of at least 65% [29]. However, to maintain the high reproducibility of retention [29], it is advisable to re-equilibrate the columns between successive runs (for ca. 20 min, while a constant current reading is the indication of equilibration). The reproducibility of retention times and separation efficiencies for hydrophobic columns has been dealt with exhaustively in other communications [9,29,30]. Here, as in the previous work, relative standard deviations were found to be below 3% (run to run) and 5% (column to column).

Most studies published on CEC to date report the separation of standard mixtures. To demonstrate the feasibility of separating analytes in complex, 'real world' samples at realistic concentrations on the monolithic columns, we extracted a steroid fraction from human pregnancy urine and dansylated the corresponding ketosteroids. The recoveries of similar extraction procedures have been investigated and are reported to be above 95% [21,23]. Fig. 7 shows the separation and detection of a complex mixture of constituents under the conditions of gradient elution (35–65% acetonitrile). 11β-Hydroxyandrosterone (peak 2), dehydroisoandrosterone (peak 3), estrone (peak 4) and androsterone (peak 5) were tentatively identified by their retention times and through the use of a sample spiking technique. The combination of CEC with MS (see below) verified these tentative identifications, in addition to tracing 16-hydroxyestrone and pregnanediol from their background-subtracted spectra.

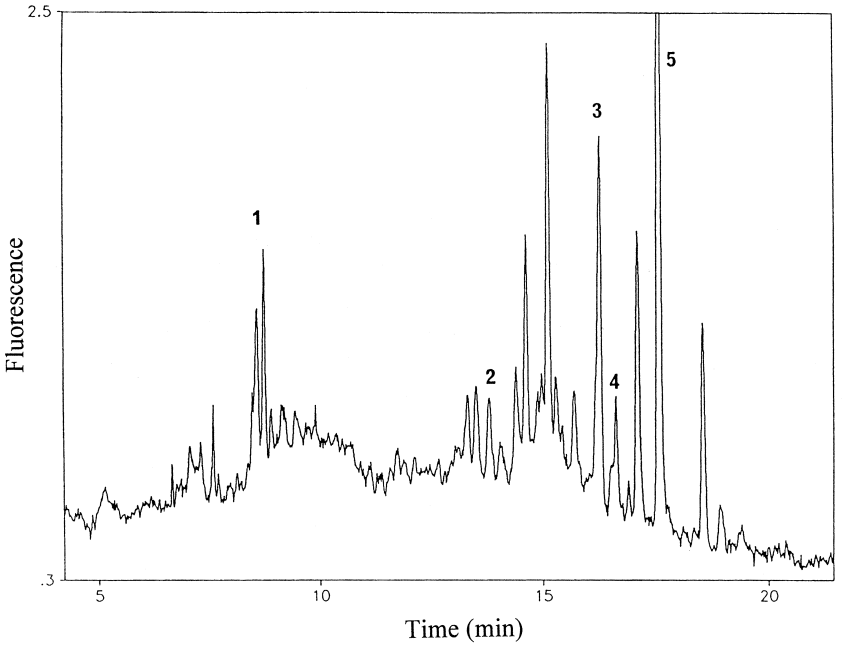


Fig. 7. Gradient electrochromatogram of derivatized urinary neutral steroids extracted from pregnancy urine. Peaks: 1 = labeling reagent, 2 = 11β-hydroxyandrosterone, 3 = dehydroisoandrosterone, 4 = estrone and 5 = spiked androsterone. Experimental conditions: same as in Fig. 6.

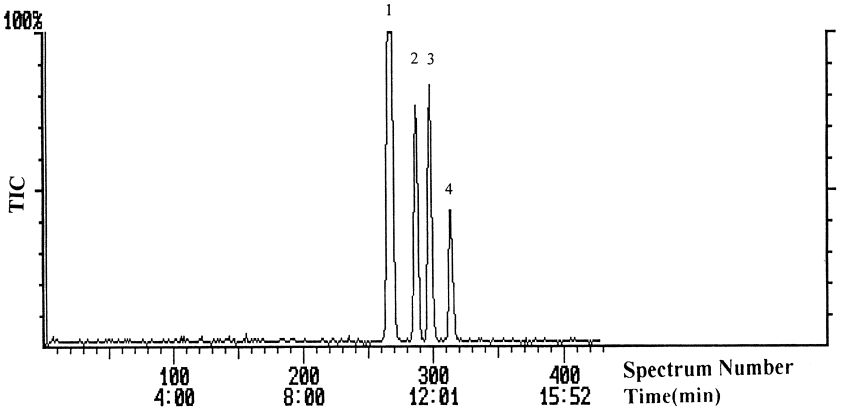


Fig. 8. CEC–ESI–MS analysis of neutral steroids. The test mixture contains 19-hydroxy-4-androstene-3,17-dione (1), 11β-hydroxy-androsterone (2), dehydroisoandrosterone and equiline (3) and androsterone (4), all at 250 μg/mL. Experimental conditions: mobile phase, acetonitrile–water–240 m*M* ammonium formate buffer (pH 3) (50:45:5, v/v/v); field strength, 350 V/cm; injection, 100 V/cm, for 30 s.

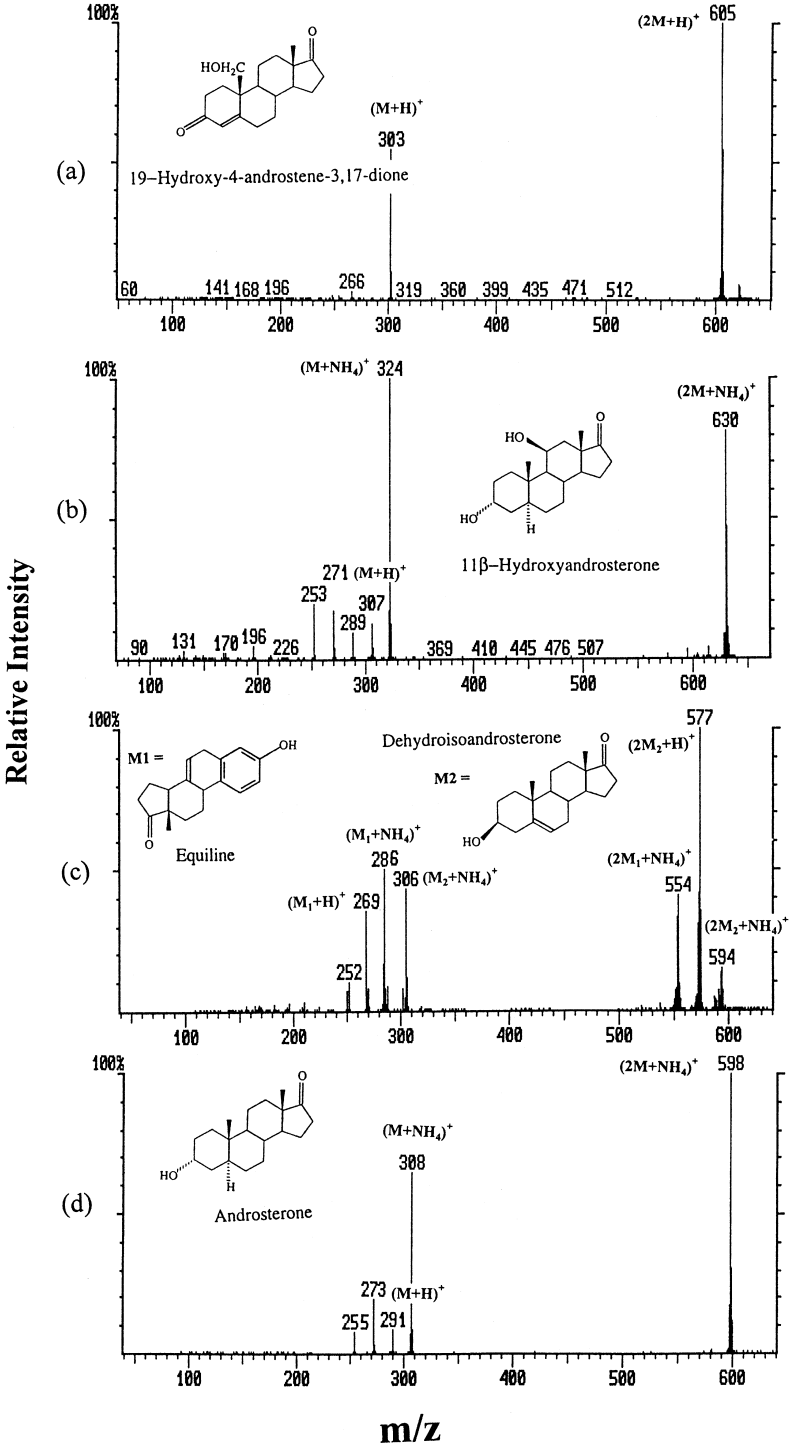


Fig. 9. Mass spectra of the neutral steroids obtained from Fig. 8. (a) Peak 1; (b) peak 2; (c) peak 3; (d) peak 4.

3.3. CEC coupling with ion-trap MS

We have found that the attachment of our CEC columns to an ion-trap instrument, as shown in Fig. 2, appears feasible, albeit at some sacrifice in detection sensitivity due to the interface using the sheath liquid. As expected, moderate band dispersion and dilution occurred as a consequence of coupling. Still, the detection limits were found in the femtomole range under full-scan conditions and at a low-femtomole to high-attomole level with the selective ion-monitoring mode. Using the dansylated steroids during CEC–electrospray MS, distinct singly charged and protonated molecular ions were observed. Regrettably, we were unable to induce a significant fragmentation of these molecules under the usual MS–MS conditions of collision-induced dissociation [17] within the ion trap. This is because fragmented dansylhydrazine carries a charge, leaving the free steroid remainder neutral and thus not observable in the spectrum. This, in addition to the already mentioned complication of observing both the derivatized and underivatized steroids in the same run, indicates the limited utility of dansylation in conjunction with CEC–MS. Consequently, we turned our attention towards the detection of underivatized steroids.

Given a suitable ionization technique, almost any modern mass spectrometer can be used as a universal detector for microcolumn separations. In addition to the dansylated ketosteroids, a variety of hydroxy steroids can be detected through the signals due to the total ion current (TIC) mass-scanning mode or, alternatively, as mass-selected ions. Fig. 8 shows a mass electrochromatogram (in the TIC mode) of a standard mixture of five underivatized neutral steroids: 19-hydroxy-4-androstene-3,17-dione,11β-hydroxyandrosterone, dehydroisoandrosterone and equiline (co-eluting in peak 3), and androsterone, all introduced at 0.25 mg/ml levels (~15 picomoles injected). The mass spectra extracted from each of the TIC-recorded peaks are shown in Fig. 9. The spectrum extracted from peak 3 unambiguously reveals that it is a composite of dehydroisoandrosterone and equiline spectra. As seen from Fig. 9, both singly charged monomers and dimers have been formed under the ESI conditions used, carrying either protons or ammonium ions. The protonated molecular ions are further observed to cleave a water molecule. Typical column efficiencies seen with CEC–MS coupling were around 100 000 theoretical plates per meter.

A shallow gradient was applied to resolve the complex mixture from pregnancy urine (results not shown). Unfortunately, the sensitivities to different steroids, which were spread fairly evenly over the elution range, varied considerably. Compared with LIF detection (as discussed above), MS detection was roughly three orders of magnitude lower. Clear mass spectra were obtained only for 16-hydroxyestrone, androsterone, and pregnanediol (data not shown) from the background-subtracted and time-averaged runs. When the mass range was extended from 650 to 1300 amu, singly charged ions for the monomer, dimer and trimer were detected for these steroids. While the volatile buffer (ammonium formate) composition and flow-rate were optimized for ESI sensitivity, only marginal improvements were achieved for the spectra of additional steroids eluting from this complex biological mixture. With the limited availability of standard steroids, additional compound identification remains a difficult task.

4. Conclusions

Macroporous acrylic matrices, provided with hydrophobic ligands, were shown here to separate neutral steroids and their biological conjugates with good efficiency. Securing high-sensitivity measurements through a fluorescent tag to the carbonyl-containing steroids and subsequent laser-induced fluorescence detection appears feasible. While coupling CEC to ion-trap mass spectrometry appears possible for identification studies, the actual analysis of trace steroids in biological fluids through CEC–MS may necessitate additional improvements in sample preconcentration and/or greater measurement sensitivity.

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