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Capillary electrophoresis with UV detection and mass spectrometry in method development for profiling metabolites of steroid hormone metabolism☆

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ABSTRACT

The aim of this study was to develop a method for comprehensive profiling of metabolites involved in mammalian steroid metabolism. The study was performed using the partial filling micellar electrokinetic chromatography (PF-MEKC) technique for determination of endogenous low-hydrophilic steroids. The detection techniques in capillary electrophoresis were UV absorption and electrospray mass spectrometry (ESI-MS). Thirteen steroids were included in the method development, and the selected were metabolites involved in major pathways of steroid biosynthesis. Although only eight of them could be separated and detected with UV, they could be identified by ESI-MS using selected ion monitoring (SIM) technique. Tandem MS spectra were also collected. UV detection was more sensitive than MS due to better separation of compounds and the selective signal sensitivity. The lowest limits of detection were 10–100 ng/mL for cortisone, corticosterone, hydrocortisone and testosterone. The other steroids could be detected at 500–1000 ng/mL. The identification of cortisone, corticosterone, hydrocortisone, estrogen and testosterone were made in patient urine samples and their concentrations were 1–40 μg/L.

1. Introduction

Mammalian steroid hormones are an important class of metabolites with diverse biochemical and physiological functions, and quantitative and comprehensive characterization of steroid hormone status in body fluids and tissues is of importance in assessment of human health. All mammalian steroid hormones are synthesized from cholesterol via pregnenolone through a series of reactions that occur in either the mitochondria or endoplasmic reticulum of the adrenal cell (Fig. 1, [1]).

Endogenous mammalian steroids undergo extensive metabolism in the human body [1] prior to their excretion in urine [2,3], where their glucuronate and sulphate conjugates dominate. The concentration of endogenous steroids such as testosterone (biosynthesis route, Fig. 1) in male urine after hydrolysis is approximately 40–60 ng/mL [4,5]. With use of a photodiode array detector in HPLC, LODs of 60–70 ng/L have been achieved [6–11]. HPLC has shown its use in many biological applications. It has been used in analysis of corticosteroids in bovine urine [12], of cosmetics [13], of liver and tissue samples [14] and human samples [15]. Estrone and β-estradiol in human fluids were found at extremely low concentrations [16]. β-Estradiol in serum has been detected between the ranges 40–270 pg/mL [2] and 5–20 pg/mL in women [17,18], and at less than 8 pg/mL in men.

The advantage of capillary electrophoresis (CE) over other chromatographic techniques is its versatility. Capillary electrophoresis was used in wide range of biological samples, such as proteins, peptides, nucleic acids, nucleotides, carbohydrates, fatty acids and drugs [19–21]. The technique is especially useful as it can be optimized for the determination of diagnostic biomarkers. The many techniques available in CE make it useful in situations where other liquid-phase separation techniques are limited or impractical. The advantages of capillary electrophoresis are high efficiency, requiring minute amounts of sample and quantitatively consuming limited amounts of reagents [18,19].

It is commonly known that even separation methods often require laborious sample pre-treatment (extraction, derivatization). However, in CE technique minor sample clean-up is required when analyte concentrations are high enough for identification. The CE-MS like the HPLC–MS techniques have some limitations [20], such as the lack of commercially available conjugated corticosteroids reducing the testing and validating possibilities. Therefore sample preparation and concentration is needed before analysis.

Compared with high-performance liquid chromatography, CE lacks sensitivity when UV detection is concerned. However,

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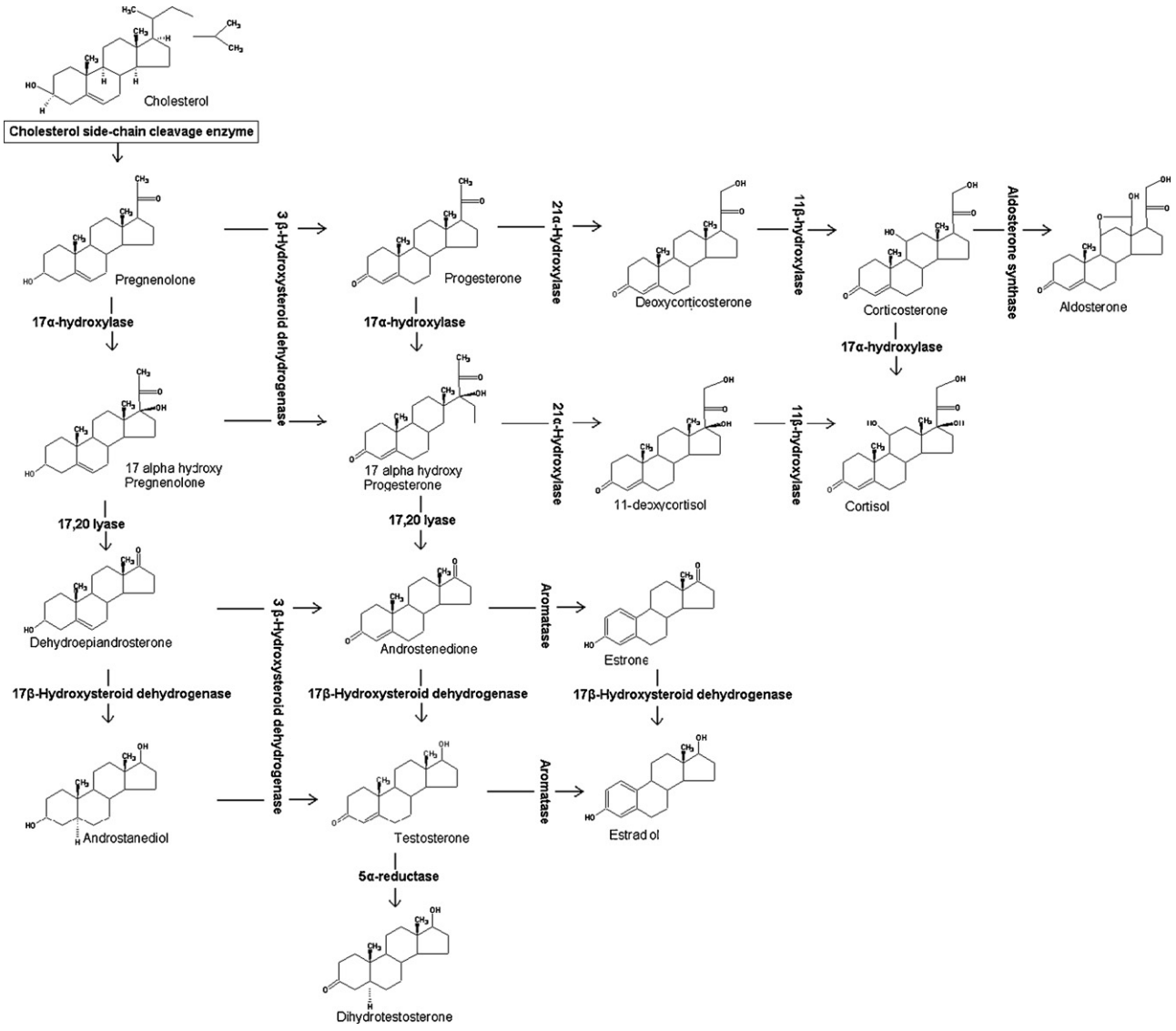

Fig. 1. Major pathway in steroid biosynthesis [http://en.wikipedia.org/wiki/Steroid_synthesis#Steroid_biosynthesis].

partial filling concentration modes are applicable in CE techniques. When coupled on-line with mass spectrometry, HPLC and CE may be considered as comparable techniques.

The application of mixed micellar electrokinetic capillary chromatography (MEKC) for the determination of the corticosteroid hormones cortisone, cortisol (hydrocortisone), and dexamethasone in serum samples has been demonstrated elsewhere [21]. After sample preparation, the serum samples were analyzed by CE. A micellar system of sodium dodecyl sulphate (SDS) and sodium cholate (SC) buffered with an organic compound provided the electrolyte solution. Spiked blank serum samples were used for the linearity testing, and limits of quantification. Patient samples were analyzed and the concentrations of cortisol and cortisone were measured. The LOD values of 20 μg/mL, which are thousand times higher than reported in other studies [22].

The analysis of endogenous adrenal steroids by CE and related techniques is challenging. Due to the low-hydrophilic nature of the corticosteroids structure, they hardly dissociate below pH 12 [23]. Therefore, the micellar electrokinetic capillary chromatography (MEKC) is used in electroseparation. In MEKC, the corticosteroid can be separated based on their mobilities in a capillary filled with electrolyte solution containing surfactants at concentrations higher than the critical micelle concentration (CMC) [24]. To be separated, electrically neutral analytes must have differing micelle–water partition coefficients, P_{mw} [25]. Because the corticosteroids have structures that are very similar to each other (Fig. 1, [26]), their separation with high resolution is required.

Earlier, excellent resolution for adrenal steroids has been achieved with mixed micelles consisting of SDS and other bile salts, *e.g.* SDS-sodium taurocholate (STC), SDS–sodium dehydrocholate (SDC) and SDS–STC/SDC. However, the MEKC applications for corticosteroids are few, and none rely on the utilization of mixed micelles, with the exception of the reports from Amundsen et al. [27–29] which used PF-MEKC-UV and PF-MEKC–ESI-MS techniques in analyses of androgens and testosterone derivatives from standards and male urine.

Clearly, exhaustive studies in this field are needed. Here we report development of a PF-MEKC method for steroid separation with CE-UV, and its application to patient urine sample in ESI-MS mode. In mass spectrometric detection the purpose was to find specific mass/charge (m/z) ions for reliable identification of the analytes in biological matrices. The metabolites used for

method development were selected from the steroid biosynthesis pathway.

2. Experimental

2.1. Instruments

2.1.1. Capillary electrophoresis

Capillary electrophoresis studies were made with a Beckman-Coulter MDQ (Beckman Instruments, CA, USA) with interfaces to a PDA detector and a triple quadrupole mass spectrometer. The 80 cm capillaries (L_{det} UV 70 cm, 50 μm i.d. × 375 μm o.d.) were used as purchased from Composite Metal Services (Werchster, UK). The total length of the capillary was chosen based on its need in CE-MS studies. The temperature control in the analyses was at +15 and +30 °C for sample storage and separation, respectively. Several different hydrodynamic injections were tested. The final analyses were completed by triplicate injections with 20 s at 2.0 psi. The separation voltage was +30 kV. Detection with UV was either at 250, 238 and 220 nm or at 247, 260 and 238 nm, depending on the separation method and the electrolyte–micelle composition (wavelength optimization was based on sensitivity). In final PF-MEKC method the current during the analyses was +17.5 μA.

New capillaries were conditioned by flushing at 20 psi pressure sequentially with 10% NH_3 in water, water and electrolyte solution for 15 min each. After run the capillary was emptied with vacuum, rinsed with water and flushed with electrolyte solution for 2, 2 and 2.5 min, respectively [29].

2.1.2. Mass spectrometer

PF-MEKC–ESI-MS experiments were performed using the CE instrument described above and a Micromass Quattro II triple quadrupole instrument (Micromass, Manchester, UK) equipped with an ESI interface. Ionization was made in positive ionization mode with a mass range of *m*/*z* 50–600 amu and *m*/*z* 10–400 amu in the MS and MS/MS modes, respectively. The nebulising gas was N_2 and collision gas Ar.

The instrument was connected through a commercial interface (Micromass) based on a coaxial sheath flow. The sheath liquid was introduced with a 1 mL syringe (Hamilton, Reno, NV, USA) with a flow rate of 60 μL/min by using a microsyringe pump (Harvard Apparatus, South Natick, MA, USA). In the PF-MEKC–MS analysis, the MEKC conditions used were according to the following modifications: The separation was performed at +30 kV and 0.5 psi back pressure. Thermosetting the capillary was not possible in PF-MEKC–ESI-MS [30]. In PF-MEKC–ESI-MS analyses, the counter-electrode lenses were removed from the ionisation chamber, since the potential of the lenses were set to zero when coupling CE with ESI-MS [30]. Several different capillary and cone voltages were tested, and they were finally set at +3.5 kV and +40 V, respectively. The source temperature was +70 °C. The flow rate of the nebulising gas was 40 L/h. No drying gas was used. Prior to analysis, the CE–ESI-MS system was tuned with a 1 μg/mL 4-androsten-3,17-dione solution introduced through the CE capillary at 1.0 psi and with a sheath liquid flow of 10 μL/min. In the SIM analyses, $[M+H]^+$ peaks were selected for each of the compounds (4-androsten-3,17-dione, estrone, β-estradiol, aldosterone, 21-deoxycortisol, hydroxycortisone, corticosterone, cortisone, progesterone, dexamethasone, 11-β-hydroxytestosterone, prednisolone and testosterone). Fluoxymesterone was used as the internal standard in extraction. The ions were monitored with a dwell time of 0.08 s and an interchannel delay of 0.20 s. In MS/MS mode, the applied collision energy was +25 kV and the pressure of the collision gas (Ar) 1.7 μbar.

2.1.3. Other instruments

The pH values of the electrolyte solution and micellar solutions were adjusted using a Denver model 20 pH meter with a combination electrode (Denver Instrument Co., CO, USA) calibrated with pH 4.00 (±0.01), 7.00 (±0.01) and 10.00 (±0.01) commercial buffers (Reagecon, Shannon, Ireland).

2.2. Chemicals

The standard chemicals were from Sigma–Aldrich (imported by YA Kemia, Helsinki, Finland).

4-Androstene-3,17-dione ($C_{19}H_{26}O_2$, MW 286.41 g/mol), testosterone ($C_{19}H_{28}O_2$, MW 288.43 g/mol), 11-β-OH testosterone ($C_{19}H_{28}O_3$, MW 304.43 g/mol), estrone ($C_{18}H_{22}O_2$, MW 270.37 g/mol), β-estradiol ($C_{18}H_{24}O_2$, MW 272.39 g/mol), aldosterone ($C_{21}H_{28}O_5$, MW 360.45 g/mol), 21-deoxycortisol ($C_{21}H_{30}O_4$, 346.5 g/mol), hydrocortisone ($C_{21}H_{30}O_5$, 362.47 g/mol), corticosterone ($C_{21}H_{30}O_4$, MW 346.47 g/mol), cortisone ($C_{21}H_{28}O_5$, MW 360.45 g/mol), progesterone ($C_{21}H_{30}O_2$, MW 314.47 g/mol), dexamethasone ($C_{22}H_{29}FO_5$, MW 392.47 g/mol) and prednisolone ($C_{21}H_{28}O_5$, MW 360.45 g/mol) were from Sigma–Aldrich Finland Oy (Helsinki, Finland). Ammonium acetate was purchased from Fluka (Buchs, Switzerland). SDS (approx. 99%) and 11β-hydroxytestosterone (4-androstene-11β,17β-diol-3-one, $C_{19}H_{28}FO_3$, MW 304.4 g/mol) were from Sigma–Aldrich (St. Louis, MO, USA) and taurocholic acid sodium salt (≥99%) from Calbiochem (La Jolla, CA, USA). Methanol was purchased from Rathburn (Walkerburn, UK), ethanol (≥99.5%) from Primalco (Rajamäki, Finland) and acetic acid from Merck (Darmstadt, Germany). All reagents were of analytical grade. HPLC quality water was produced with a Milli-Q A10 system (Millipore, Bedford, MA, USA).

Helix pomatia juice was obtained from BioSepra (Cergy-Saint-Christophe, France). The solution contained 100 000 Fishman Units (FU) of β-glucuronidase and 1 000 000 Roy Units (RU) of sulphatase in 1 mL. The enzyme activity was not separately checked but the amount declared by the supplier was used as a basis for dosage.

2.3. Preparation of standard solutions

The stock solutions of 0.5–6.0 mg/mL of 4-androstene-3,17-dione, estrane, β-estradiol, aldosterone testosterone, hydrocortisone, corticosterone, cortisone, progesterone, dexamethasone, prednisolone, 21-deoxycortisol and fluoxymesterone were prepared in ethanol and stored at −4 °C in 1.5 mL glass vessels. Concentrations of the working solutions of the standards were 0.2–50 ng/μL for each analyte made either in the mixture of methanol – 20 mM ammonium acetate (pH 9.68) or in that of methanol–water (both in 50:50, v/v). The electrolyte solution was optimized by concentration and pH, and it was 20 mM ammonium acetate (pH 9.68). The pH was adjusted with 25% ammonium hydroxide (ad. vol. 150 μL). The electrolyte solution was filtered with a 0.45 μm MF filter (Millipore, Ireland) and stored at +4 °C in a glass vessel. The micelle stock solutions were made of 100 mM SDS in 20 mM ammonium acetate by varying the pH range between 6.8 and 10.2. They were stored at room temperature in a glass container, and not filtered before use. The stock solution of 36.8 mM sodium taurocholate in 20 mM ammonium acetate (pH 9.68) was stored at +4 °C in a glass container, and not filtered before use. The final mixed micellar solution consisted of 29.3 mM SDS and 1.1 mM sodium taurocholate in 20 mM ammonium acetate at pH 9.68. Before, MEKC runs, all solutions were kept in ultrasonic bath for 15 min at room temperature. The sheath liquid in PF-MEKC–ESI-MS was a mixture of methanol–water (50:50, v/v) containing 0.1% formic acid.

2.4. Preparation of electrolyte solutions

The electrolyte solutions used were made of ammonium acetate and the micelle solution from SDS and taurocholate. Combinations of pH (6.8–10.2) and the volumes of the different solutions were used in optimization of the partial filled MEKC in the capillary. In total, 20 different electrolyte compositions in ESI-MS were tested.

2.5. Preparation of urine samples and a serum standard

2.5.1. Urine samples

The patient urine samples were obtained from the University Hospital of Helsinki (Helsinki, Finland). The patients (ages 30–75) were suffering from high-blood sugar. In addition, they had excreted amounts of catecholamine metabolites (NMN, MN and MOMA). The samples were 24-h urine collected into vessels containing 5 mL of 6 mol/L HCl. The samples had been frozen (−80 °C) for 1 year, and after obtaining the samples they were kept at −20 °C in the laboratory until used. In the enzyme hydrolyses, amounts of 3, 5, 7, 10 or 12 μl of *Helix pomatia* juice were tested to find the optimal enzyme concentration in real samples. The experiments were done with a urine blank, made in water, containing each corticosteroid. The total volumes of the samples were 1000 μL, corresponding to a steroid concentration of 5 μM and enzyme activities of 300–1200 FU and 3000–12 000 RU. The mixtures were incubated at +40 °C temperature for 4 h. No reagent was used to halt the reaction.

After hydrolyses the samples were pre-treated with solid-phase extraction (SPE) by using either a Strata X (Phenomenex, Torrance, CA, USA) or an Oasis (HLB, Waters, Taunton, MA, USA) according to the instructions of the manufacturers. First, the materials were conditioned with 1 mL volumes of methanol and water, followed by introduction of the hydrolyzed patient samples. Secondly, the materials were washed with methanol–water solution (5:95, v/v) and the analytes were eluted with 2 mL of methanol. After evaporation to dryness (+60 °C) the samples were dissolved into 1 mL methanol.

The samples were screened immediately by CE. The final quantitative results were made with CE-ESI-MS. The samples were kept at +4 °C before use. In the CE instrument, during the sequential analyses, the samples were stored at +15 °C. The urine samples were prepared in duplicate.

2.5.2. Serum samples

Serum samples were kept at −80 °C at Technical Research Centre of Finland when received from the University Hospital of Helsinki (Helsinki, Finland) and during the analysis period at −20 °C until used. The samples were stored for more than one year. Spiked serum samples (50 μL) were tested both without protein precipitation and after precipitation with methanol. The samples were diluted with Milli-Q water and thereafter proteins were precipitated with 50 μL methanol. The supernatant was used as the sample in PF-MEKC–ESI-MS analyses.

2.6. PF-MEKC validation

The CE separation of the analytes and their monitoring with a photodiode array detector was validated with nine different standard mixtures, in which the concentrations varied between the range of 0.1–5 mg/L of 4-androstene-3,17-dione, estrane, β-estradiol, aldosterone, testosterone, 21-deoxycortisol, hydrocortisone, corticosterone, cortisone, progesterone, dexamethasone and prednisolone (Fig. 2). Occasionally, one solution containing hydrocortisone, testosterone and 4-androstene-3,17-dione was injected three times in every measurement period in order to testing the performance of the CE-ESI-MS analyses.

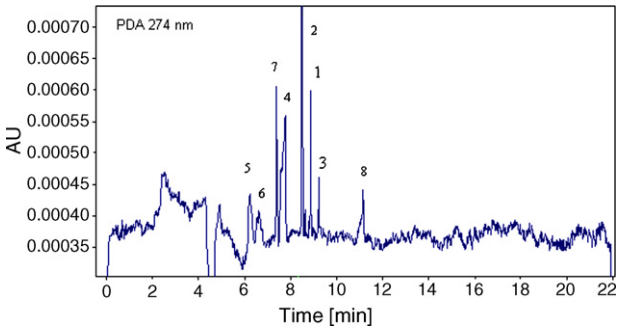

Fig. 2. PF-MEKC-UV electropherogram of a standard containing 2.8 ng/mL. Compounds as in Fig. 1: testosterone (1), 4-androstene-3,17-dione (2), β-estradiol (3), hydrocortisone (4), cortisone (5), corticosterone (6), aldosterone (7) and dexamethasone (8); voltage is +30 kV; injection with 5 psi for 5 s; capillary dimensions (L_{tot} 80 cm, L_{det} 70 cm, 50 μm i.d.); detection with UV at 247 nm. Electrolyte solution: 20 mM ammonium acetate (pH 9.68).

3. Results and discussion

In this study, a partial filling micellar capillary electrokinetic (PF-MEKC) method was optimized with capillary electrophoresis in order to separate and to identify the steroid compounds in urine and serum. The analytes were the compounds of the major pathway in steroid biosynthesis (Fig. 1). The results showed that the best resolution for the analytes was obtained by using 20 mM ammonium acetate in water at pH 9.68 with PF zone of 36.8 mM taurocholate and 29.5 mM SDS (Fig. 2). Similarly, the pH of the micellar solution used for partial filling of the capillary to enhance the detection of the analytes sensitivity was optimized [26,27].

In PF-MEKC with UV and ESI-MS detections methanol was used in the samples to improve the solubility of the steroids. It was found that the apparent mobility was $6.62 \times 10^{-8}\ V^{-1}\ m^2\ s^{-1}$ in UV detection. This was noticed from the increase of absorption between electrolyte solution and micelle zone at 18.3 min with apparent mobility of $1.70 \times 10^{-8}\ V^{-1}\ m^2\ s^{-1}$. In ESI-MS technique the methanol marker was detected at around 1 min and 16 min in the methanol and micelle phase, respectively, due to the forward pressure during the analysis.

The analytical performance of the PF-MEKC-UV and PF-MEKC–ESI-MS techniques was depending on the analytes used to estimate it. However, it was noticed that in UV system it was better due to the temperature control. With five repetitions, the RSD values of cortisone, corticosterone and hydrocortisone were 2.1–2.6%, those of testosterones, estrone, β-estradiol 0.5–1.5% and the others 2.4–2.7% depending on their concentration. In PF-MEKC–ESI-MS system the RSD values of their migration times were between 1 and 3%. The linear ranges used in PF-MEKC–ESI-MS analyses were between 50 ng/mL and 30 μg/mL.

LOD values obtained with UV detection were 10–100 ng/mL for cortisone, corticosterone, hydrocortisone and testosterone. The other steroids could be detected at 500–1000 ng/mL. With ESI-MS detection they were 0.5–1 μg/mL. β-Estradiol and estrone could only be detected at above 1 μg/mL level. LOQ values were dependent on the SPE treatment and they were from 100 ng/mL (testosterone) to 10 mg/mL (β-estradiol) in PF-MEKC–ESI-MS.

3.1. CE-UV

In PF-MEKC-UV method the capillary was filled with the basic electrolyte, micelle solution prepared by SDS and taurocholate, a

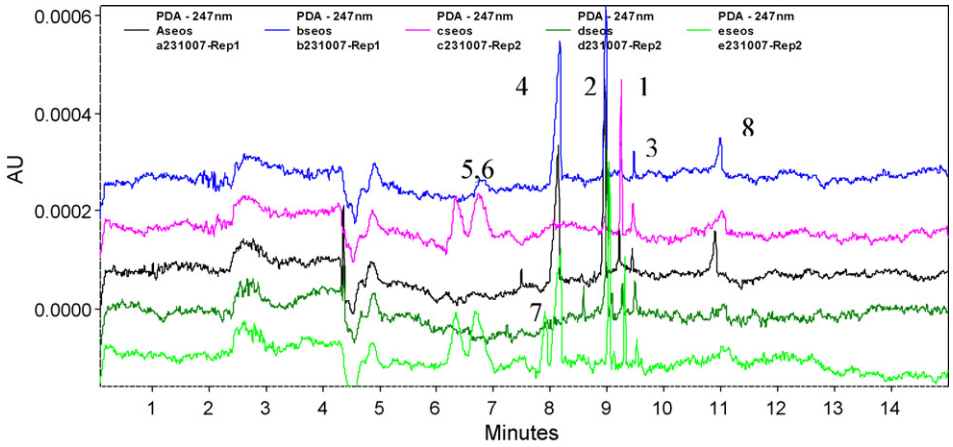

Fig. 3. PF-MEKC-UV electropherogram of five different analyte mixtures at 50 ng/mL level containing (1) testosterone, (2) 4-androstene-3,17-dione, (3) β-estradiol, (4) hydrocortisone, (5) cortisone, (6) corticosterone, (7) aldosterone and (8) dexamethasone. Experimental conditions: voltage +30 kV; sample injection with 5 psi for 5 s; capillary dimensions (L_{tot} 80 cm, L_{det} 70 cm, 50 μm i.d.); detection with UV at 247 nm. Electrolyte solution: 20 mM ammonium acetate (pH 9.68). Other details in Section 2.

sample, a micelle solution and finally another plug of the electrolyte. The purpose of the discontinuous separation solvent is that the analytes, when reaching the micelle zone will interact with the micelles differently from each other, and these differences that depend not on their size and charge but also on their low-hydrophilic nature. The micelle zone was optimized to be 50.3 mm (6.3% of the total capillary length, calculated with Beckman-Coulter CE Expert Lite). In the PF-MEKC-UV analyses the concentrations of the standards in their mixtures were from 0.2 to 4 μg/mL. The studied corticosteroids profiles are presented in Figs. 2 and 3 under optimized PF-MEKC-UV conditions. Currents in the analyses were +6.5 μA. Eight of the analytes could be separated from each other, although the other migrated together. In spite of this, all compounds present in the injected standard could be identified with spiking technique in the electropherograms: A concentration of 1 μg/mL of each compound was added to the sample solution. The hydrocortisone, cortisone and corticosterone analytes were problematic, since their separation could not be obtained at high concentrations due to the broad overlapping sample zone. Furthermore, estrane and β-estradiol showed lower UV response compared to that of the other corticosteroids. The results showed that increases of over 200-fold in the UV abundances of the main analytes were achieved.

The concentration effect of the PF-MEKC-UV technique was tested with five different compositions of standard sample mixtures. It was noticed that even low ng/mL levels of analytes could be detected with a signal-to-noise ratio above 3. The only exceptions were estrane and β-estradiol, as mentioned above. The sensitivity was not enhanced but instead the sample zones were broadened for the two estrogens when 11% of the total volume of the capillary volume was filled with the sample. Our results showed that their behaviour was exceptional compared to that of the other corticosteroids. Comparison with literature results could not be done, since our PF-MEKC study is the only one for analysis of estrane and β-estradiol. UV detection was not a good choice to identify the analytes in real samples, particularly after using the unselective sorbent in SPE. The reason for this was the many UV absorbing compounds existing after hydrolysis step. The SPE treatment did not reduce their existence in the final sample. Fig. 4 shows typi-

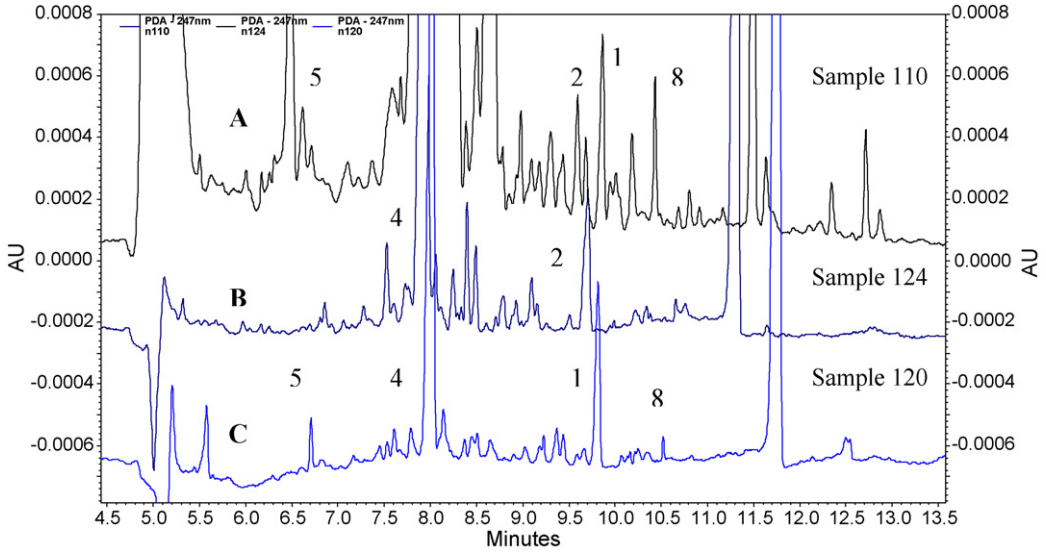

Fig. 4. PF-MEKC-UV electropherograms of three urine samples: (A) 110, (B) 124 and (C) 120 (without dilution). Samples pre-treated with SPE. Peaks detected with spiking from the samples were (1) testosterone, (2) 4-androstene-3,17-dione, (4) hydrocortisone, (5) cortisone and (8) dexamethasone. Experimental conditions: voltage +30 kV; sample injection with 5 psi for 5 s; capillary dimensions (L_{tot} 80 cm, L_{det} 70 cm, 50 μm i.d.); detection with UV at 247 nm. Electrolyte solution: 20 mM ammonium acetate (pH 9.68).

Table 1
CE-ESI-MS analyses with positive ionization mode

Compound	Main MS fragments (amu)	Main MS/MS fragments (amu)
Testosterone	343.2 (70%), 329.3 (35%), 311 (100%), 289.2 (25%)	176 (20%), 109 (100%), 97 (98%)
Prednisolone	361.3 (30%), 325.2 (40%), 214.1 (100%), 163.0 (45%)	361.2 (20%), 342.9 (35%), 170.9 (55%), 147 (100%)
Hydroxycortisone	363.2 (100%), 214.1 (60%), 158.2 (20%)	363.2 (100%)[a]
4-Androstene-3,17-dione	309.2 (50%), 287.2 (85%), 214.2 (75%), 181.3 (90%), 149.4 (100%)	199 (10%), 109 (80%), 97 (100%)
Cortisone	361.2 (60%), 309.2 (50%), 60.3 (100%)	361 (20%), 163.1 (100%)
Costicosterone	347.2 (80%), 60.3 (100%)	347.2, 329.2 (100%), 311 (20%)
11-β-OH-Testosterone	327.3 (40%), 305.2 (100%)	173 (80%), 147 (80%), 121 (100%)
Estrone[b]	271.2 (40%), 236.2, 214.2 (100%)	236, 214 (93%), 79 (100%), 60 (100%)
21-Deoxycortisol	347.2 (20%), 214.2 (20%), 70.3 (40%)	311 (90%), 269 (70%), 121.1 (100%)
Progesterone	369.2 (80%), 337.2 (100%), 315.2 (20%)	109 (100%), 97 (70%), 72 (65%)
β-Estradiol[c]	272.2 (100%), no other	272.2 (100%), no other
Dexamethasone	393.2 (20%)	372.8 (50%), 355.3 (60%), 237.1 (100%)
Aldosterone	361.2 (100%), 34.23 (15%)	343 (35%), 315 (25%), 299.1 (50%), 182.5 (50%), 108.9 (100%)

[a] Ionization with positive ionization and negative ionization is poor.
[b] Fragmentation also with negative ionization technique is poor (negative ionization: fragments in MS: $(M-H)^-$ 269.2 amu (100%), 213.2 amu; MS/MS: 269.2 amu (100%)); shield liquid methanol–water–0.1% NH_3.
[c] Fragmentation also with negative ionization technique is poor (negative ionization: fragments in MS: $(M-H)^-$ 271.2 amu (100%), 212.2 amu; MS/MS: 271.2 amu (100%)); shield liquid methanol–water–0.1% NH_3.

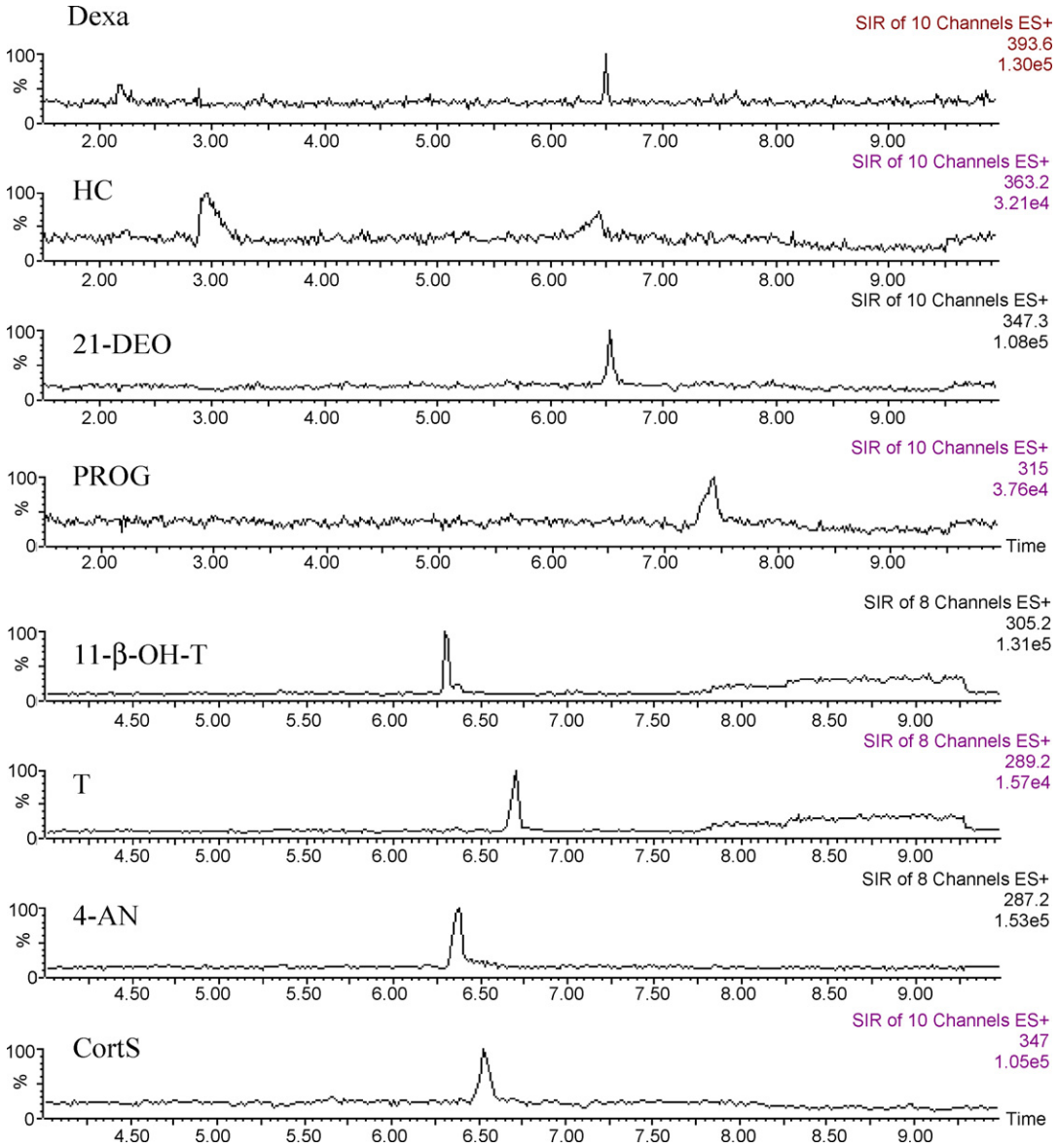

Fig. 5. PF-MEKC–ESI-MS (SIM) chromatograms of a 1 μg/mL standard mixture. Mass fragments are listed in Table 2. Conditions are explained in Section 2. Dexa, dexamethasone; HC, hydrocortisone; 21-DEO, 21-deoxycortisol; PROG, progesterone; 11-β-OH-T, 11-β-OH-testosterone; T, testosterone; 4-AN, 4-androsteone; CortS, corticosterone. Electrolyte solution: 20 mM ammonium acetate (pH 9.68). Other details in Section 2.

cal electropherograms obtained from the pretreated urine samples. Finding more selectivity in UV detection was not studied further due to the fact that the major aim of the study was to optimize mass spectrometry for selective identification of the analytes. That being the case, the matrix compounds were not supposed to disturb the analysis.

3.2. PF-MEKC–ESI-MS

The PF-MEKC separation method that was optimized with UV was used with minor modifications in the MS detection. The only technical improvement was the use of forward pressure during the analysis to speed up the sample flow to MS. Therefore, the pressure was optimized on basis of the shield flow rate and the stability of the separation current. When the micelle phase is partially filled in the capillary, the analytes enter the MS well before the surfactants reach the MS system as described previously. The analysis was stopped at 13 min before the micelles reach the MS. Currents in the analyses were +17.5 μA.

The detection sensitivity in CE-UV was improved owing to the technical arrangements of the instrumentation, which produced a uniform electric field through the capillary. However, in PF-MEKC–MS studies the discontinuous electric field caused differences in corticosteroid ionization and their dissolution in the shield liquid making the sensitivity of some analytes was very low. In ESI-MS, especially estrane and β-estradiol fragmentation, gave mass fragments of low intensity, and could not be analyzed at an ng/mL level. With CE-MS the sample peaks were broader than those obtained with the CE-UV method. This is a problem since β-estradiol in human fluids appears at very low concentrations [1]. In women's serum in ranges 40–270 pg/mL [2] or 5–20 pg/mL [2,3], and in men lower than 8 pg/mL [3]. Therefore, approximately 1 mL of serum is needed to analyse the particular compounds with the PF-MEKC–ESI-MS technique developed. Identification of the steroid hormones was assured by determining the ESI-MS spectra of the

Table 2
Results calculated from PF-MEKC–ESI-MS (SIM) electropherograms peaks

Samples	Concentrations (μg/mL)	Compounds
Urines		
Sample 20	0.37	Cortisone
MOMA 28 μmol	Below 0.02	Hydrocortisone
Sample 120		
NMN 1.2 μmol	1.53	Cortisone
MN 1.1 μmol	Below 0.02	Hydrocortisone
Sample 123		
NMN 0.8 μmol	0.78	Cortisone
MN 0.5 μmol	Below 0.02	Hydrocortisone
Sample 999		
NMN 1.8 μmol	0.90	Cortisone
MN 12.8 μmol	0.03	Hydrocortisone
	4.75	Progesterone
	2.34	Aldosterone
Sample 11	18.8	Testosterone
Sample 95	2.18	4-Androstene-3,17-dione
NMN 0.4 μmol	8.57	Cortisone
MN 0.3 μmol	Below 0.02	Hydrocortisone
Sample 76	16.8	4-Androstene-3,17-dione
MOMA 28 μmol		
Sample 84	8.0	Testosterone
MOMA 26 μmol	3.76	4-Androstene-3,17-dione
Sample 110	4.40 (added 4.70)	Cortisone
	Below 0.02 (added 0.05)	Hydrocortisone
Serum	5.04 (added 5.0)	Hydrocortisone
Spiked sample	9.37 (added 9.5)	Cortisone
	1.27 (added 1.4)	Corticosterone

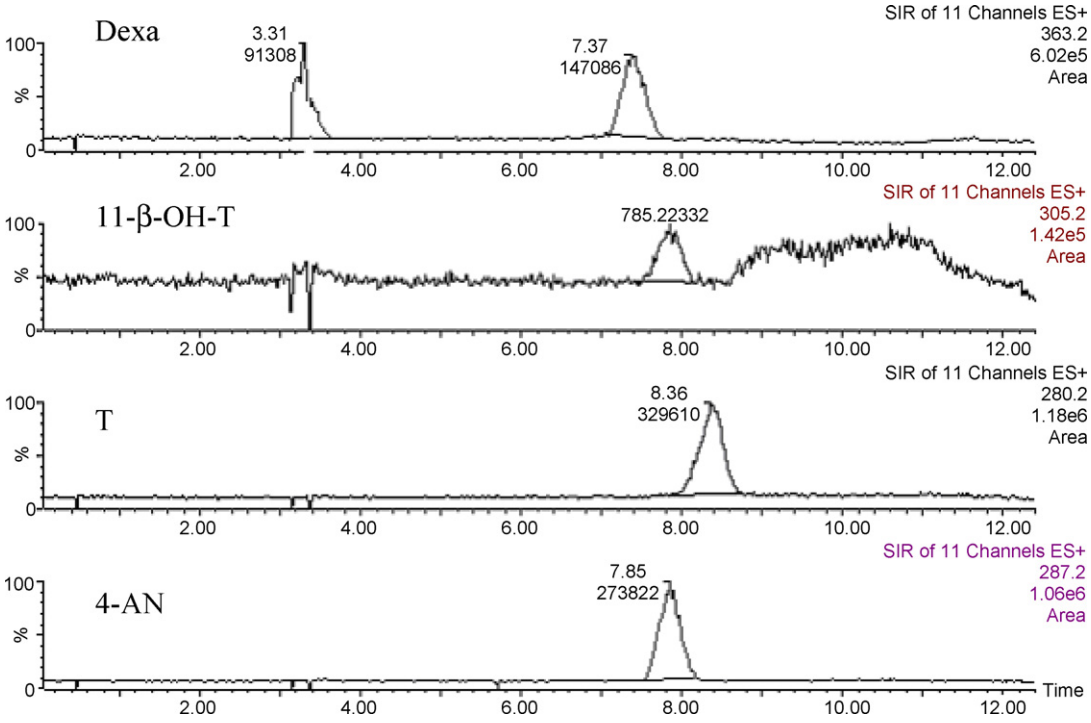

Fig. 6. PF-MEKC–ESI-MS (SIM) electropherograms of the urine sample 123. The *m*/*z* fragments are presented in Table 2. Electrolyte solution: 20 mM ammonium acetate (pH 9.68).

analytes by direct infusion of the standard solutions. The main mass fragments for all the analytes based on measurements with positive ionization mode are listed in Table 1. SIM was used for sensitive detection. In TIC profile, eight characteristic ions were selectively chosen based on the monitored analytes (Fig. 5). It is possible that increases of more than 100-fold in the sensitivity can be obtained when using SIM techniques.

The final optimized method was used for analyses of selected urine samples (male and female) and of a spiked serum sample. The samples were pretreated as described in Section 2. Volumes of 200 and 40 μl of urine and serum supernatants were used for the analyses, respectively. The calculations were made with response factors obtained by using the analyte concentrations listed in Table 2. An example of the SIM chromatograms is shown in Fig. 6. The patient urine samples contained notable amounts of cortisone, testosterone and 4-androstene-3,17-dione. Some of the samples also contained minor amounts of hydrocortisone (around LOQ). The profile of one urine sample was very different from the others: It contained cortisone and hydrocortisone, but also significant amounts of progesterone and aldosterone (Table 2).

4. Conclusions

PF-MEKC-UV and PF-MEKC–ESI-MS methods based on sequential introduction of a small volume of pseudostationary phase made of sodium dodecyl sulphate and taurocholate into capillary has been presented. The method allowed quite rapid analyses of the metabolites of steroid hormone synthesis. The separation of eight compounds was completed in 12 min with PF-MEKC-UV and the separation of all in 9 min with PF-MEKC–ESI-MS (SIM). The potential of the method was shown by determining the analytes from hydrolyzed patient urines and one spiked human serum. We expect that the method can be applied to profiling tissue- and cell-specific analytes, and this will be one of our future goals.

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