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Research Article

Simultaneous determination of nine endogenous steroids in human urine by polymeric-mixed micelle capillary electrophoresis

A new CE system based on the use of polymeric-mixed micelles (cholic acid, SDS and the poloxamine Tetronic® 1107) was developed for the simultaneous determination of nine steroids in human urine. This method allows the baseline separation and quantitation of cortisol, androstenedione, estriol, dehydroepiandrosterone sulfate, testosterone, dehydroepiandrosterone, estrone, progesterone and estradiol in less than 25 min showing to be sensitive enough to detect low concentrations of these steroids in urine samples (5–45 ng/mL). The optimized electrophoretic conditions were performed using a 50 cm × 75 μm capillary, 18 kV, 25°C, with 44 mM cholic acid, 10 mM SDS, 0.05% w/v tetronic® 1107, 2.5% v/v methanol, 2.5% v/v tetrahydrofuran in 5 mM borate – 5 mM phosphate buffer (pH = 8.0) as a background electrolyte and a dual 210/254 UV-detection. The method can simultaneously determine 0.1–120 μg/mL, which corresponds to 5–6000 ng/mL of steroids in 2 mL urine. The recoveries ranged between 82.4 and 101.5%. Due to its simplicity, speed, accuracy and reliability, the proposed method could be a potential alternative to the traditional methodologies used with clinical purposes.

Keywords:
CE / Micellar electrokinetic capillary chromatography / Polymeric micelle / Steroids
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1 Introduction

Steroid hormones are an important class of compounds with diverse biochemical and physiological functions and their quantitation is important in the assessment of the state of human health.

Vertebrate steroids comprise the sex steroids (androgens, estrogens and progestagens), corticosteroids (glucocorticoids and mineralocorticoids) and anabolic steroids.

Additionally, numerous synthetic steroids have been used as therapeutic agents in the hormonal replacement like in menopause and other hormonal diseases, as contraceptives, anti-inflammatory or immunosuppressant agents [1–3].

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Abbreviations: Δ_4, androstenedione; **CA,** cholic acid; **Cort,** cortisol; **DHEA,** dehydroepiandrosterone; **E2,** estradiol; **E3,** estriol; **E1,** estrone; **IACE,** immunoaffinity CE; **LLE,** liquid–liquid extraction; **Pg,** progesterone; **PSP,** pseudo-stationary phases; **SDHEA,** dehydroepiandrosterone sulfate; **sβ-CD,** sulfated β-cyclodextrin; **To,** testosterone

Steroids are synthesized from cholesterol *via* pregnenolone through a long biosynthetic pathway (Fig. 1) and the production of these substances is concentrated in ovaries, adrenal cortex, testes and placenta. They undergo extensive metabolism in the body prior to their excretion in urine in the forms of glucuronate and sulfate conjugate metabolites [4, 5].

Biochemical studies have demonstrated that there are characteristic changes in the concentrations of steroids both in plasma and urine and these measurements have proved to be equally useful. However, while the urinary measurement of steroids usually indicates the amount secreted during the collection period, the concentration in plasma may be altered by rapid fluctuations in steroid levels [6]. A great interest exists in the development of rapid and reliable methods for detection and/or determination of steroids in several matrices in different areas such as biomedical applications (metabolic disorders, pregnancy, cancer and bone diseases), pharmaceuticals quality control and doping control of hormonal drugs [7]. In addition, there are evidences that endogenous sex steroids and cortisol (Cort) are related to the etiology of intrahepatic cholestasis of pregnancy, although there is a controversy of which of them are altered [8–11].

To achieve the exact determination of steroids at low concentrations in biofluids it is mandatory to consider, first,

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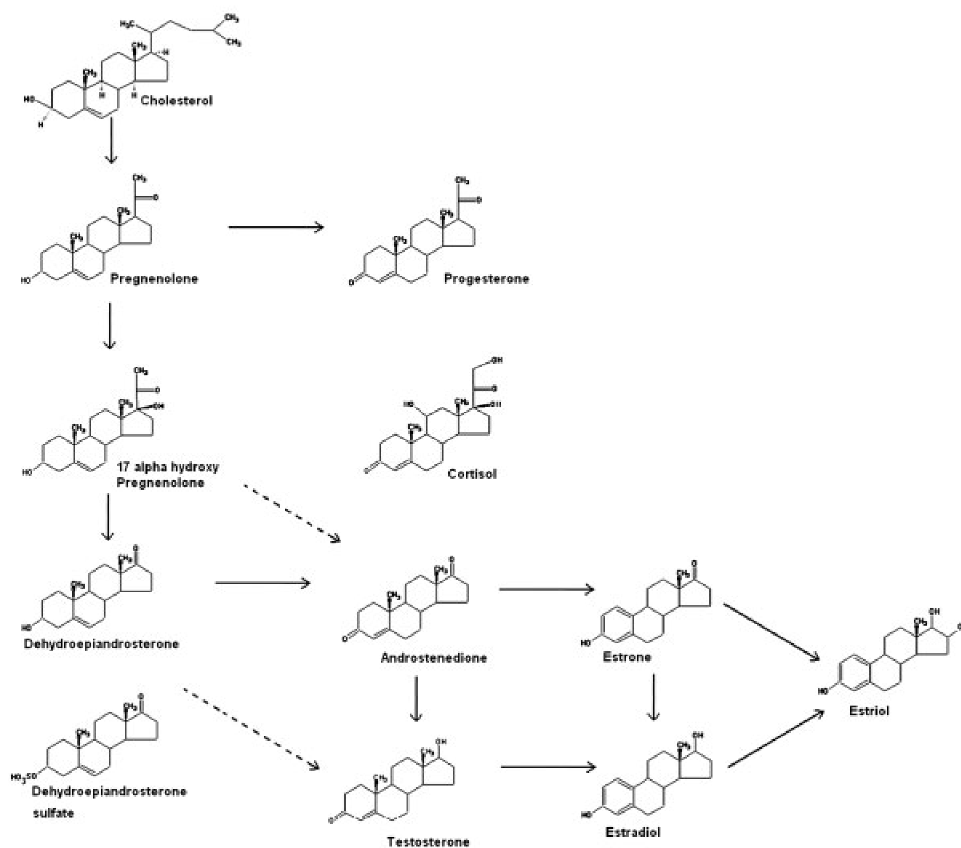

Figure 1. Pathway in the steroid biosynthesis (http://en.wikipedia.org/wiki/Steroid_synthesis#Steroid_biosynthesis).

that the sample preparation technique must provide a high yield and selectivity and, second, that the analytical method should be reliable and simple to be used in routine analysis in clinical laboratories.

Usually, an urine sample preparation must include enzymatic hydrolysis with β-glucuronidase/sulfatase, purification and preconcentration of the samples before analysis.

Liquid–liquid extraction (LLE) has been traditionally used for the analysis of steroids because it provides high recoveries and adequate selectivity. The main drawbacks of LLE are derived from emulsion formation and long time duration of the procedure. SPE using cartridges packed with bonded silica C18 have also been carried out for clean-up of complex matrices with the advantages of high reproducibility and recovery, high selectivity, speed and sample volumes smaller than those employed in LLE without the risk of emulsion formation and amenable to be automated [7, 12].

For screening purposes, RIA and enzyme immunoassay are widely used in the analysis of steroids [13, 14]. Although these immunological techniques are attractive because of simplicity, speed and high sensitivity they present many disadvantages such as high cross-reactivity with a remarkable overestimation because antibodies are poly-reactive and can recognize and interact with similar epitopes of what was originally intended to react, high-grade lot-to-lot variation of antibodies, false positive and negative data and the additional disadvantage of the necessity to analyze hormones individually [1, 6].

For confirmatory testing, chromatographic techniques such as GC and LC, especially those coupled to MS, are usually preferred [2, 15, 16]. However, these approaches require qualified operators, sophisticated instrumentation, high costs and complex sample preparations often with derivatization steps prior to injection. In the case of the classical LC, the separation is slow and incomplete. As a result of these factors, the method is not yet satisfactory from the point of view of speed, simplicity and routine application [6].

In the last decade, CE with its different modes of operation, has proven to provide great utility in the analysis of different types of compounds and to be an attractive alternative to traditional methodologies due to its high efficiency, reduced sample volume and reagent consumption, short analysis time, wide rage of analysis and the possibility of making changes in the electrolyte composition in order to separate a wide range of hydrophobic and hydrophilic compounds in complex matrices [17]. Electrolyte additives have been used to modify the eletrophoretic mobility of analytes for better separation. In particular, the development of MEKC, in which micelles are added to the electrolyte as pseudo-stationary phases (PSP), has greatly expanded the utility of the technique because it combines features of both LC and CE techniques. The combination of both separation

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mechanisms results in a powerful tool that makes possible to separate complex mixtures of analytes. The varying rates of partition between the complex analyte–micelle lead to excellent selectivity in separation [18, 19]. Furthermore, selectivity can be enhanced by the use of mixed particles as PSP. Organic solvents can also be added to the electrolyte to affect the rate of inclusion complex formation; however, high concentration of an organic solvent could reduce the number of micelles. An important strategy to solve this problem is the use of polymeric micelles used as PSP that can introduce various advantages such as better stability of micelles at high percentages of organic solvents, they do not have a CMC and they also display larger cores than surfactant micelles leading to higher solubilization capacity of hydrophobic compounds compared to regular micelles [18, 20, 21]. Although the potential limitations of the polymer micelle EKC such as poor reproducibility of the polymer synthesis, it is possible that its addition to the PSP improves EKC performance and utility [22].

MEECK is another mode of CE in which microemulsion is the PSP [17]. Recently, we have developed an MEEKC system using a novel microemulsion suitable for the simultaneous determination of seven natural and synthetic estrogens in pharmaceuticals [23]. However, the resolution of the system is not enough for the complete and simultaneous separation of estrogens, progestagens and androgens in biological fluids.

A further improvement on the CE assay is their application to chip-based systems [24]. The advantages of microscale CE and microchip are in progress; however, sensitivity and selectivity of detection are relatively weak points [25]. To overcome these problems one of the most promising preconcentration technique is immunoaffinity CE (IACE) which combines the advantages of both immunoassays and CE [25]. IACE with or without microchip systems has been used to analyze hormones in biological fluids [24, 26] with remarkable results compared to other immunoaffinity procedures such as the absence of either false positive or negative results [26]. However, although nanotechnology based in IACE possesses many advantages, is still a promising technique for clinical laboratories since there are no commercially available concentrators and they must be in-house prepared [26].

In addition to IACE, there are many contributions of other research groups to the separation of different steroids using MECK systems.

Analytical methods for the analysis of certain estrogens using MEKC systems with SDS (MEKC-SDS) or, cholic acid (CA), (MEKC-CA) [1, 27–29] as surfactants with organic modifiers and/or cyclodextrins have been reported. Although these separations are fast and consume low sample and reagent volumes, analysis of steroids in biological fluids requires highly sensitive and selective methods because of the extremely low concentration of the analytes and presence of interferences in the samples [4].

Even when the separation of many steroids has been reported, in some cases, detection could not be successful when the monitoring is performed in real samples [27]. To improve detection limits of steroids using MECK with UV detection, different strategies were implemented like z-type capillary flow cell [30], stacking with CDs [31, 32], dynamic pH junction sweeping [33], partial filling concentration [4], pressure assisted field amplified sample injection [34], preconcentration with gold nanoparticles and sweeping MEKC [35] and analyte focusing by micelle collapse [36]. Although the contribution of these innovative methods is remarkable, they were not always simple enough to develop in a routine laboratory [5, 35].

Moreover, some steroids are difficult to resolve being necessary to change the detection system under the same operational condition [1].

However, a strict comparison between methods is complicated by the fact that the different authors have tested different mixtures of steroids in their methodologies.

On the other hand, in the pharmaceutical technology field, one of the most recent drug delivery agents are polymeric micelles using poloxamines like Tetronic® 1107 (Fig. 2). This compound is a nanoscopic structure formed by amphiphilic block copolymers with a remarkable advantage which is the presence of two tertiary amine groups in the center of the molecule that confers a dual behavior: temperature and pH sensitiveness. With respect to pH sensitivity, it is known that in a more basic medium the micelles were larger and the size distribution more homogeneous [20].

To our knowledge, the employment of this copolymer Tetronic® as a PSP in CE systems has not been previously reported.

This paper describes the development and evaluation of a sensitive capillary electrophoretic method for the determination of nine steroids: Cort, androstenedione (Δ_4), estriol (E3), dehydroepiandrosterone sulfate (SDHEA), testosterone (To), dehydroepiandrosterone (DHEA), estrone (E1), progesterone (Pg) and estradiol (E2) in human urine.

The proposed method involves a combination of two surfactant micelles (CA and SDS) plus a polymeric micelle (Tetronic®1107) and a mixture of organic solvents to allow a highly selective separation. Sample pretreatment was performed by enzymatic hydrolysis followed by a SPE using C18 cartridges.

As far as we know, the simultaneous determination of the steroids studied in our work has not been analyzed before by MEKC.

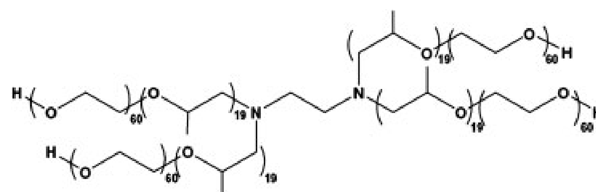

Figure 2. Chemical structure of poloxamine Tetronic®1107.

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2 Materials and methods

2.1 Chemicals and reagents

Cortisol 4-pregnene-11β,17α,21-triol-3,20-dione (Cort), Androstenedione 4-androstene-3,17-dione (Δ_4), estriol (1,3,5(10)-estratiene-3,16α,17-βtriol) (E3), dehydroepiandrosterone 5-androsten-3β-ol-17-one (DHEA), testosterone 4-androsten-17β-hydroxy-ol-3-one (To), dehydroepiandrosterone 5-androsten-3β-ol-17-one sulfate (SDHEA), estrone (1,3,5(10)-estratien-3-ol-17-one) (E1), progesterone 4-pregnene-3,20-dione (Pg), estradiol (1,3,5(10)-estratiene-3,17-β-diol) (E2), β-cyclodextrin sulfate sodium salt, SDS, sodium cholate hydrate (CA), β-glucuronidase from *Helix pomatia* (*type H-2*) were purchased from Sigma (St. Louis, MO, USA). Tetronic® 1107 was a gift from BASF Corporation (Florham Park, NJ, USA). Sodium monohydrogen phosphate, sodium borate 10-hydrate, tetrahydrofuran, ethanol and methanol were HPLC grade and supplied by E. Merck (Darmstadt, Germany). Sep-Pak C18 SPE cartridge (Waters, USA). Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, Dubuque, IA, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, Westboro, MA, USA) and degassed before use.

2.2 Instrumentation and electrophoretic conditions

Analysis was carried out with a P/ACE™ MDQ CE system (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 50 cm (40 cm length to the detector) × 75 μm id, were used. The capillary temperature was maintained at 25°C, and UV detection was set at two different wavelengths 210 and 254 nm. Samples were injected under 0.5 psi pressure for 3 s and electrophoretic system was operated under positive polarity and a constant voltage of 18 kV.

The separation was performed by MEKC system consisting of 44 mM CA, 10 mM SDS, 0.05% w/v tetronic® 1107, 2.5% v/v methanol, 2.5% v/v tetrahydrofuran and 5 mM borate- 5 mM phosphate buffer (pH = 8.0) as a BGE.

A new capillary was pretreated rinsing for 5 min with 0.5 M potassium hydroxide, 2 min with 0.1 M potassium hydroxide, 5 min with water and 10 min with BGE. Between runs, the capillary was conditioned during 1 min with 0.1 M potassium hydroxide, 1 min with water and BGE for 3 min. At the end of the day, the capillary was flushed during 3 min with 0.1 M potassium hydroxide and 5 min with water. In all cases a pressure of 50 psi, was applied in the equipment.

2.3 Stock and standard solutions

Stock solution of nine steroids containing E1, E2 and E3 at 1 mg/mL, To and Pg at 2 mg/mL, DHEA and SDHEA at 9 mg/mL, and Cort at 6 mg/mL were prepared by dissolving in methanol.

Standard solutions were obtained by appropriate dilution with 3% sulfate-β-cyclodextrin in 5 mM borate – 5 mM phosphate buffer (pH = 8.0) at a final concentration of 2 μg/mL (E1, E2 and E3), 4 μg/mL (To and Pg), 18 μg/mL (DHEA and SDHEA) and 12 μg/mL (Cort).

2.4 Sample preparation

Urine samples of 24 h were obtained from healthy volunteers. β-glucuronidase/sulfatase of 50 μL from *Helix pomatia* (*type H-2*) was added to 2 mL of urine sample. Enzymatic hydrolysis was carried out at 55°C for 1 h. After hydrolysis, the sample was passed through a Sep-Pak C18 SPE cartridge conditioning with 2 mL methanol and 4 mL deionized water, and eluted with 2 mL methanol.

The eluate was evaporated to dryness and redissolved in 100 μL of diluent composed of 3% w/v sulfate-β-cyclodextrin in 5 mM borate-5 mM phosphate (pH = 8.0) with 20% of methanol allowing a 20-fold concentration.

2.5 Quantification and evaluation methods

The quantification of steroids was performed using calibration curves. Concentrations for calibration were chosen based on the levels of urinary steroids encountered in normal urine. As the original urine samples without concentration contain undetectable amounts of steroids using this methodology, they were chosen as the blanks. Calibration curves were obtained using blank samples as matrix, which were spiked with steroid standards to a final concentration of 0.1–120 μg/mL range which corresponds to 5–6000 ng/mL range of steroids in urine. Accuracy was evaluated by means of a recovery assay. The recovery assays were carried out by spiking steroids to blank samples at three different levels in triplicate corresponding to the upper limit, lower limit and middle point of the calibration curve.

The LOD and LOQ values in urine were determined at S/N ratios of 3:1 and 10:1, respectively. These values were calculated at the maximum wavelength of each steroid: 210 nm for E1, E2, E3, DHEA and SDHEA and 254 nm for Cort, Δ_4, Pg and To. Table 1 presents LOD range between 0.03 and 0.3 μg/mL (corresponding to 1.5–15 ng/mL in urine) and LOQ range between 0.1 and 0.9 μg/mL (corresponding to 5–45 ng/mL in urine),

The stability of steroids in urine was evaluated under different conditions. We stored urine samples for 24 h at room temperature, 8 and −20°C and after that, they were processed immediately. Additionally, a fresh urine sample was extracted and analyzed immediately. Stability was expressed in terms of % of concentration of each steroid recovered compared to the concentration of the fresh sample analyzed (reference value). The acceptance criterion was set as no more than 10% loss of recovery with respect to the reference value.

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Table 1. Linearity, LOD and LOQ of the steroids

	LOD (μg/mL)	LOQ (μg/mL)	Linearity range (μg/mL)	r^2
Cortisol	0.08	0.3	0.3–100.0	0.990
Δ_4	0.06	0.2	0.2–60.0	0.995
E3	0.04	0.1	0.1–15.0	0.991
SDHEA	0.3	0.9	0.9–120.0	0.996
To	0.06	0.2	0.2–45.0	0.993
DHEA	0.2	0.7	0.7–120.0	0.994
E1	0.03	0.1	0.1–15.0	0.990
Pg	0.09	0.3	0.3–45.0	0.990
E2	0.06	0.2	0.2–15.0	0.987

3 Results and discussion

3.1 Optimization of the electrophoretic system

3.1.1 Type and concentration of micelles

Traditional MEKC systems with SDS micelles were not successful to separate the present steroids. The system containing 50 mM SDS in borate:phosphate buffer at pH 8.0 *e.g.* was not able to resolve E1 from DHEA and Pg from To and E3 (Fig. 3A). Replacing SDS by CA, another common micelle former, led to shorter analysis time, but not to a complete separation of the analytes, as Pg and DHEA, and E2 and SDHEA remained unresolved (Fig. 3B). Unsatisfactory results were also obtained with mixtures of these two surfactants (SDS and CA in the range of 5 and 50 mM). Here the best result was obtained with 50 mM CA and 10 mM SDS, but separation of E2 and Pg was still unsuccessful (Fig. 3C). Therefore, we decided to apply a ternary MEKC system with another tenside in addition-Tetronic® 1107 – assuming that it might implement a different selectivity into the system due to its chemically different properties (it is a copolymer containing ether and aliphatic amino groups).

The conventional meaning of polymer micelle is a micelle whose monomers are polymerized so that they are covalently bonded to each other. The CMC for these types of polymer micelles is zero and they do not disaggregate upon the addition of large amounts of organic solvent [22, 37].

In contrast, the poloxamine employed in this work is not a polymer micelle *per se*. It is a block copolymer that because of its chemical composition and properties can more accurately be described as a 4-tailed surfactant that will both self-aggregate to form micelles on its own and will also form mixed micellar aggregates with SDS and CA. As such, it has a non-zero CMC and there would be certain organic solvents that could be used to cause poloxamine micelles or poloxamine-SDS-CA mixed micelles to disaggregate [21].

A closer examination of the interaction between the nine steroids and the final PSP might lead to better understand their separation. It is known that solute interactions with the PSP occur *via* a variety of mechanisms such as surface adsorption, comicellization or partitioning into the hydrophobic core of the micelles. Depending upon their nature, the analytes may reside in several regions on and/or within the micelles [38]. Indeed, although the hydrophobic interaction between the solute and the final PSP is the major driving force in chromatographic separations, the elution order of the steroids under study does not seem to be governed entirely by the hydrophobicity. For example, in spite of that Cort, which is the less hydrophobic compound of the series (log *P*: 0.5), is the first eluted and E2, the most hydrophobic (log *P*: 4.2) is the last one, it was observed that certain steroids such as DHEA (log *P*: 3.0) retain longer than compounds with highly hydrophobic character such as E3 and To (log *P*: 3.6). The same phenomenon was observed by Akbay *et al.*, who studied analytes with log *P* between 2.2 and 3.9 (similar than the steroids of the present work) using different polymeric surfactants in MEKC [37].

In the discussion of the hydrophobicity of a compound often log *P* values are taken as characteristics. However, this is a rough measure only, and many examples are found where log *P* values do not well correlate with retention factors in MEKC. A striking example is presented here, as we have observed that the log *k* data from MECK with SDS (ranged between 5 and 50 mM) of the steroids assayed show an inferior correlation with log *P* (r^2: 0.1966). It was reported that the unique structure of the micelle formed by CA favors the separation of hydrophobic compounds compared to SDS [39]. However, although log *k*−log *P* correlation was increased (r^2: 0.4264) using CA, this surfactant did not enable the complete separation of the steroids assayed. Similarly, the separation was improved using both surfactants together and the log *k*−log *P* correlation was increased as well (r^2: 0.5154). However, the critical pair E2–Pg, remained unresolved even with different combinations of concentrations of both surfactants. Although the finally used PSP (SDS–CA-copolymer) was successful for a complete steroid separation, log *k*−log *P* correlation was still very low (r^2: 0.5320). This is a clear indication that hydrophobicity as expressed by log *P* is not the appropriate characteristic to describe the selectivity of the ternary MEKC system under consideration; indeed our results show that more complex interactions must be responsible for the retention of the analytes [37].

Since other authors described that micellization of Tetronic® is favored at pH > 8 [20], we have taken into account the solubility of the solute as another important parameter. To evaluate the possible interaction of the copolymer with the critical pair E2–Pg, we compared the solubilization of these steroids in the PSP composed of 50 mM CA: 10 mM SDS upon the addition of the copolymer. This procedure was carried using the method reported by Lövgen *et al.* and Gonzalez Lopez *et al.* [21, 40]. The latter authors demonstrated that the pair E2–Pg could be solubilized independently of each other [40] (simultaneous solubilizations of some steroids were, on the other hand, dependant on each other). We have observed that E2 solubility increased by 92% (moles of steroid *per* moles of total surfactant: 0.013–0.025)

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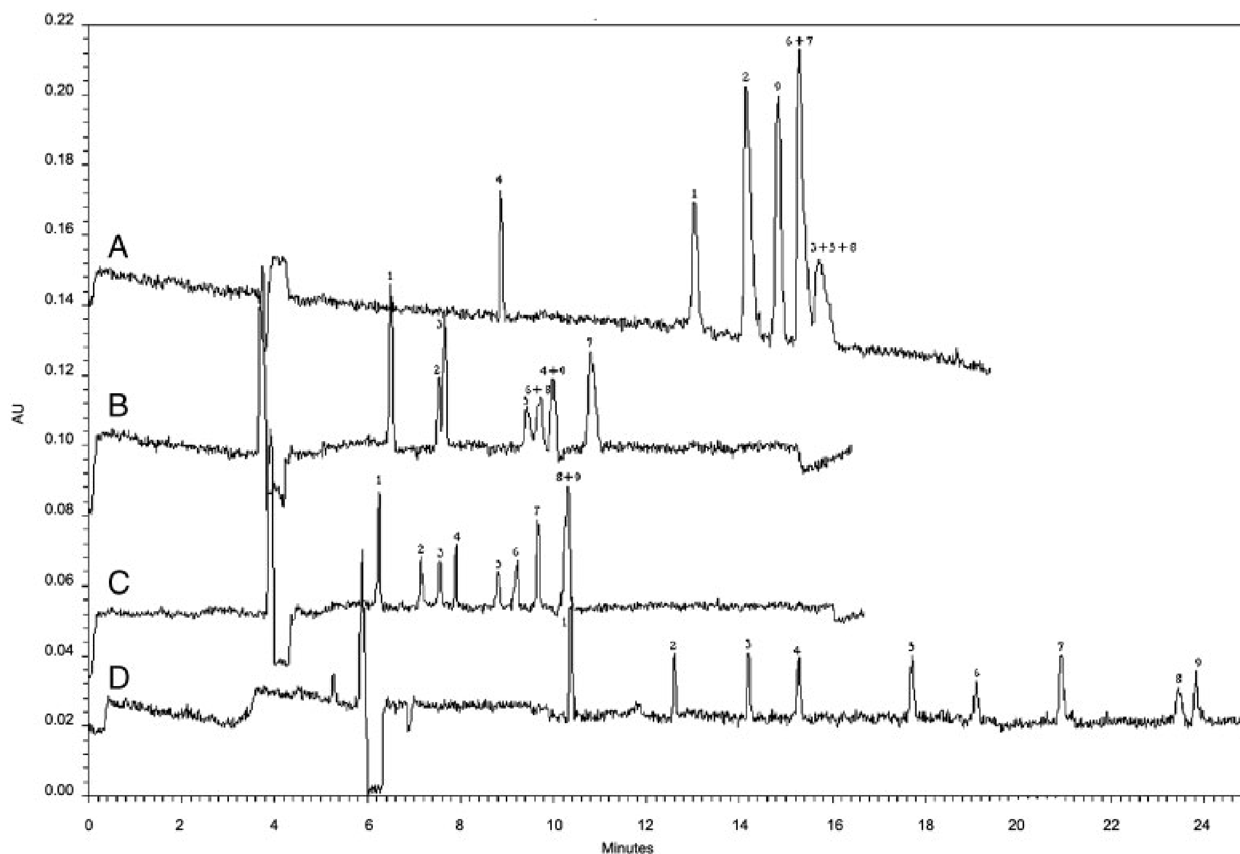

Figure 3. (A) Electropherogram of nine steroids standard by MEKC-SDS (50 mM SDS in borate:phosphate buffer pH 8.0, λ 210 nm). 1: Cort, 2:Δ_4, 3: E3, 4: SDHEA, 5: To, 6: DHEA, 7: E1, 8: Pg, and 9: E2. (B) Electropherogram of nine steroids standard by MEKC-CA (50 mM CA in borate: phosphate buffer pH 8.0, λ 210 nm). 1: Cort, 2:Δ_4, 3: E3, 4: SDHEA, 5: To, 6: DHEA, 7: E1, 8: Pg, and 9: E2. (C) Electropherogram of nine steroids standard by MEKC-CA-SDS (50 mM CA, 10 mM SDS in borate: phosphate buffer pH 8.0, λ 210 nm). 1: Cort, 2:Δ_4, 3: E3, 4: SDHEA, 5: To, 6: DHEA, 7: E1, 8: Pg, 9: E2. (D) Electropherogram of nine steroids standard and a real urine sample by MEKC-CA-SDS-poloxamine (50 mM CA, 10 mM SDS, 0.05% poloxamine in borate: phosphate buffer pH 8.0 with 2.5% ME, 2.5% THF, λ 210 nm). 1: Cort, 2:Δ_4, 3: E3, 4: SDHEA, 5: To, 6: DHEA, 7: E1, 8: Pg, and 9: E2.

with the addition of the copolymer, whereas Pg showed the same solubility in both PSP (moles of steroid *per* moles of total surfactant: 0.008) and diminished with respect to E2. This observation suggests that the higher and differential solubilization between E2 and Pg caused by the copolymer influences their separation in a positive manner. As a consequence, it was possible to obtain the complete resolution of the nine steroids when 0.05% of Tetronic®1107 was constituent of the separation buffer (Fig. 3D).

A closer insight into the types on interactions could be obtained by the determination of the solvation parameters based on the linear free energy relationship model. However, it was the goal of this paper to work out MEKC conditions for the separation of the analytes of interest rather than to carry out a more detailed study on solute descriptors; this will be the topic of our future work.

3.1.2 Solvent effect

It is known that organic solvents modulate steroids separation and their addition helps to the resolution of complex mixtures since they enlarge the elution window, allowing better separation [1]. We have investigated the electrolyte modification with: 5% methanol, 5% ACN, 5% THF, 5% ethanol, 2.5% THF/2.5% ACN and 2.5% THF/2.5% methanol. The parameters of good analytical quality such as peak shape, stability of the baseline and precision were obtained adding a mixture of 2.5% THF/2.5% methanol in the electrolyte.

3.1.3 pH and buffer concentration effect

The pH of the buffer presented a pronounced effect on the separation selectivity as it affected both, analyte ionization and the velocity of EOF generated [41]. In this study, the influence of pH was evaluated in the range between 7.0 and 12.0, and the best pH value to achieve a complete baseline separation of the analytes was found to be 8.0.

In order to reduce the microamperage developed during the run, the concentration of the buffer was reduced to 5 mM of borate and 5 mM of phosphate without changing the resolution capacity of the system.

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3.2 Optimization of the sample preparation

Since most of the endogenous steroids analyzed in this study were excreted in urine at very low concentrations either free or conjugated as glucuronides or sulfate forms, a previous hydrolysis with β-glucuronidase/sulfatase was carried out.

Sample preconcentration can provide an alternative approach to sensitivity enhancement. A combination of techniques such as SPE and LLE with CE can be used to improve sensitivity. Since different authors have reported the use of organic solvents such as hexane, dichloromethane or diethyl ether to extract urine steroids [6, 15, 42], we tried the addition of different solvents to the sample preparation. Although hexane was the best solvent for this purpose, recoveries were low and C18 SPE was tried for better extraction. Despite the main drawbacks of SPE procedures, being time consuming and requiring many steps, with lot-to-lot variations of the C18 material and possibly impaired accuracy and precision of the results [43], this methodology is one of the most common procedure used for sample preparation because it is very simple, less expensive and it provides good analyte recoveries and adequate selectivity [44].

Different elution solvents from SPE columns were also evaluated: hexane, diethyl ether and methanol. The cleaner electropherograms with better recoveries were obtained with methanol elution.

3.3 Optimization of the sample injection

Preliminary data showed that sulphated β-cyclodextrin (sβ-CD) was a good sample matrix component for providing a pronounced stacking effect and subsequent enhancement in sensitivity for detecting hydrophobic compound as well as being an excellent solubilizing agent [31]. Concentrations of 1–5% w/v sβ-CD in diluent were assayed to determine optimal concentration able to improve peak sharpening.

The injection time was also increased from 2 to 5 s in consecutive runs. We observed a much better S/N ratio when steroids were dissolved with 3% w/v sβ-CD in diluent and during 3 s injection and thus, improving the sensitivity of the method.

3.4 Method evaluation

After optimization of the method, the evaluation procedure was accomplished according to the bioanalytical environment.

Linearity, LOD and LOQ, precision, accuracy and stability were determined in urine samples.

Linearity was performed at five different concentration levels in the range between 0.1 and 120 μg/mL. The average correlation coefficient was 0.992. Values of LOQ and LOD are given in Table 1.

The intra- and interday precision of the results obtained for analysis of steroids in urine was tested by analyzing three concentration levels of the calibration curve at low level (QC1), middle level (QC2) and high level (QC3) of each steroid studied (Table 2).

Accuracy was evaluated from recovery studies of samples of steroids in urine at three concentration levels. The mean steroid recoveries were between 82.4 and 87.7% for low levels (RSD intraday 5.5–10.0, RSD interday 9.0–11.2), 86.5–92.6% for middle levels (RSD intraday 0.8–5.0, RSD interday 5.3–10.2) and 98.2–101.5% for high levels (RSD intraday 2.3–7.1, RSD interday 7.2–10.7). In every case, acceptable precision values for biological samples were accomplished.

The storage stability data have shown that it is necessary to process the sample within the day of reception to avoid the loss of concentration of steroids.

3.5 Application to urine samples

To demonstrate the applicability of the developed analytical method, we analyzed endogenous steroids, in a real urine sample of healthy female volunteer and the results are shown in Fig. 4. The quantitation of each steroid was determined using the calibration curve at the maximum wavelength absorbed for each one. A detection and identification process of these steroids based on the retention times and diode array detector was carried out. The UV spectra of each peak in the electropherogram was stored and subsequently compared with standards. In each case, the spectra were normalized and overlaid. Because of the sample pre-treatment with β-glucuronidase/sulphatase, it was not expected to find SDHEA in the sample after complete hydrolysis.

Table 2. Precision of the method

	Spiked levels (μg/mL)			Precision (RSD)					
				Intraday ($n=3$)			Interday ($n=9$)[a]		
	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
Cortisol	5.0	40.0	80.0	4.1	2.7	1.6	4.0	10.0	4.2
Δ_4	3.0	20.0	45.0	3.9	3.7	2.0	3.8	9.0	4.6
E3	1.0	6.0	12.0	11.7	7.0	1.0	11.0	10.7	1.0
SDHEA	6.0	50.0	100.0	5.9	5.3	7.7	7.8	8.1	9.3
To	2.0	15.0	30.0	4.5	3.6	2.8	3.9	10.0	4.6
DHEA	6.0	50.0	100.0	3.7	6.7	4.4	8.2	8.9	4.0
E1	1.0	6.0	12.0	10.0	4.5	0.6	12.0	14.9	1.0
Pg	2.0	20.0	35.0	7.2	3.8	5.7	16.0	11.3	5.4
E2	1.0	6.0	12.0	4.5	4.0	5.7	17.0	6.7	6.2

a) Obtained on three different days.

QC_1: low quality control, QC_2: medium quality control, QC_3: high quality control.

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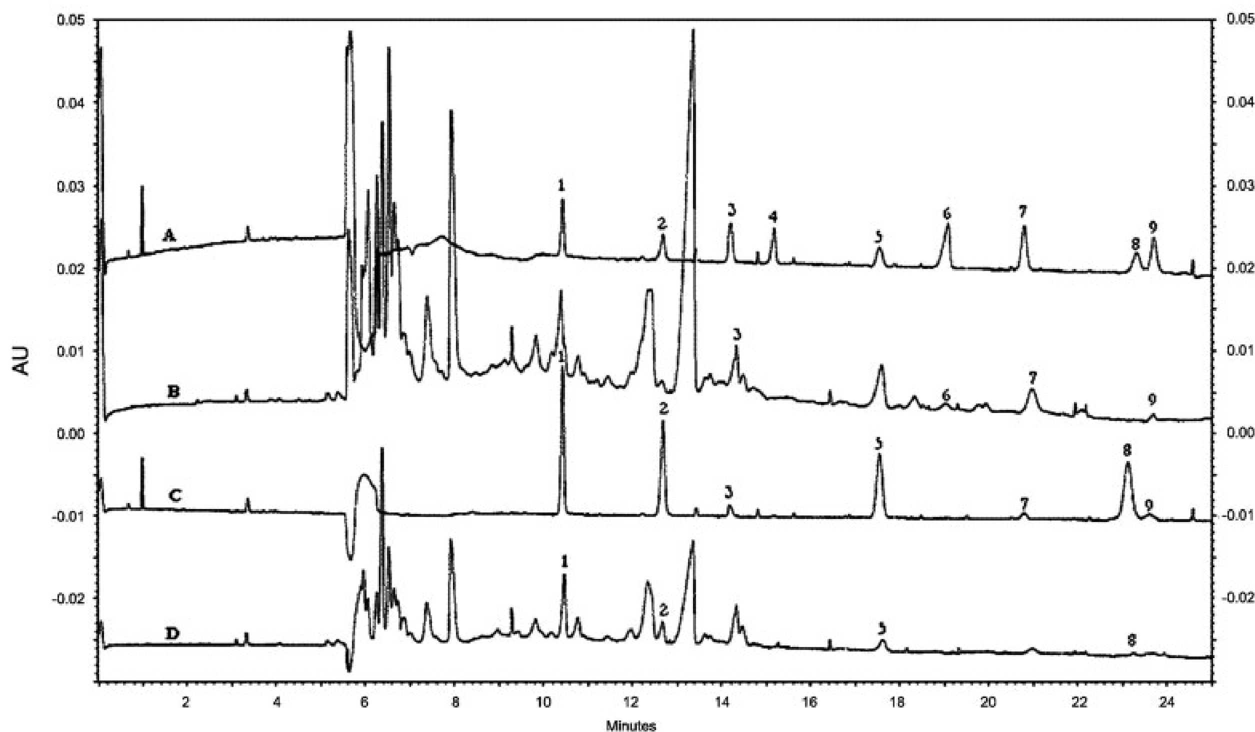

Figure 4. A: nine steroids standard at 210 nm. B: urine sample at 210 nm. C: nine steroids standard at 254 nm. D: urine sample at 254 nm.

4 Concluding remarks

A very simple and rapid capillary electrophoretic method is proposed for the quantitative and simultaneous analysis of nine steroids in urine samples with good precision and accuracy using a new polymeric-mixed micelle system based on the employment of two surfactant agents (SDS and CA) plus a polymeric micelle (the copolymer Tetronic®1107). A simple extraction and preconcentration procedure was also optimized increasing the efficiency of the clean-up procedure with a 20-fold concentration of the original sample. This method was shown to be sensitive enough to detect low concentrations of these steroids in urine samples. It can also serve to detect urinary changes tending to evaluate not only hormonal disorders or alteration in menstrual cycle but also to support more information on the role of those steroids in hepatobiliar diseases like intrahepatic cholestasis of pregnancy.

Therefore, the present electrophoretic method is proposed as an alternative assay for the determination of nine endogenous steroids amenable to be introduced in routine laboratories and clinical studies.

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