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Analysis of anabolic steroids by partial filling micellar electrokinetic capillary chromatography and electrospray mass spectrometry

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Abstract

A partial filling micellar electrokinetic capillary chromatography (PF-MEKC) separation of six anabolic androgenic steroids (androstenedione, metandienone, fluoxymesterone, methyltestosterone, 17-epimetandienone and testosterone) is introduced. The method utilises a mixed micellar solution consisting of sodium dodecyl sulphate (SDS) and sodium taurocholate. The analytes are detected with a photodiode array detector at 247 nm wavelength. Methyltestosterone is used as internal standard. The detection limits were 39 μg/L for androstenedione, 40 μg/L for testosterone, 45 μg/L for fluoxymesterone, 45–90 μg/L for 17-epimetandienone, 59 μg/L for methyltestosterone and 90 μg/L for metandienone. Linear correlation between concentration (0.1–5.0 mg/L) and detector response was obtained with r^2 of 0.994 for fluoxymesterone, 0.998 for 17-epimetandienone and 0.999 for androstenedione, metandienone and testosterone. In addition, ionisation of the investigated compounds in electrospray mass spectrometry (ESI-MS) was studied in positive ion mode. The most intense signal (100%) was the protonated molecular ion $[M + H]^+$, except for 17-epimetandienone, which gave its strongest signal at *m*/*z* corresponding to $[M - H_2O + H]^+$. Finally, separation and identification of fluoxymesterone, androstenedione and testosterone by PF-MEKC–ESI-MS is described. This is the first use of PF-MEKC and PF-MEKC–ESI-MS assays for anabolic androgenic steroids.

Keywords: Doping analysis; Partial filling micellar electrokinetic chromatography; Steroids; Hormones

1. Introduction

Steroid hormones have a hydrophobic tetracyclic perhydro-1,2-cyclopentanophenanthrene ring in their structure. They are often classified by their biological activity into androgens, oestrogens, progestogens, corticosteroids and mineralocorticoids. The naturally occurring male hormone testosterone (17β-hydroxy-androst-4-en-3-one) is an androgen. It is mainly excreted from the testis, but also from the adrenocortex. Natural plasma testosterone concentrations range from 10 to 35 nmol/L in healthy adult male down to <2 nmol/L in women and children [1].

Testosterone has both anabolic and androgenic effects in the human body. Anabolic effects stimulate nitrogen fixation and increased protein synthesis [2]. Androgens, including testosterone, are required life-long by males. They are involved in prenatal androgenic sexual differentiation, sexual maturation and persistence of fertility throughout life. They also act on a number of reproductive and non-reproductive target tissues, including bone, skeletal muscle (myotrophic action), brain, etc. [1].

Natural anabolic steroids, such as testosterone and androstenedione (androst-4-ene-3,17-dione), undergo extensive metabolism in the human body before their excretion in urine. Phase-I reactions usually convert the steroid by enzymatically catalysed reactions into more polar structures to inactivate it and facilitate its elimination from the body. Phase-II reactions act to couple the anabolic steroid or its Phase-I metabolite with glucuronic acid or sulphate [3]. Conjugates are more hydrophilic and have lower affinity for plasma proteins than the intact steroid. Deconjugation is also possible, however, and free steroids can be found in urine [4].

The rapid metabolism of testosterone, and the need for longer-acting and orally active compounds with lower androgenicity and higher anabolic activity, has led to the

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development of more than 100 synthetic steroids [2]. These derivatives of testosterone are called anabolic androgenic steroids (AAS). AAS are clinically used to treat muscle-wasting disorders, bone marrow failures, growth retardation, alcoholic hepatitis and damaged myocardium [2]. Because of their myotrophic action, AAS including testosterone have been widely used by athletes to improve athletic performance.

The Medical Commission of the International Olympic Committee (IOC) prohibits the use of anabolic androgenic steroids. The list of prohibited anabolic agents includes androstenedione, fluoxymesterone, metandienone, methyltestosterone, testosterone and many other related substances [5]. The misuse of AAS is monitored by detection of the parent steroids and (or) their metabolites in urine. The IOC criteria set for many anabolic agents and their metabolites are that a urine sample is considered positive when the measured intensity is above the signal intensity of a control urine enriched to a nominal concentration of 2 μg/L [6]. Doping with endogenous steroids is nevertheless the most challenging issue facing doping testing; when cleverly administered, they are very difficult to detect. Determining if an athlete has used endogenous anabolic steroids may involve finding an abnormal ratio of two compounds at issue in urine (e.g. testosterone/epitestosterone >6) [7]. Besides the control of abuse, steroid determinations are important for medical diagnosis of diseases related to steroid disturbances, for example adrenal problems [2,8,9].

In this study, six structurally similar anabolic androgenic steroids (androstenedione, fluoxymesterone, metandienone, methyltestosterone, testosterone and 17-epimetandienone, Fig. 1a–f) were separated by partial filling micellar electrokinetic capillary chromatography (PF-MEKC). The detection of the investigated compounds was performed by photodiode array detection (DAD). In addition, ionisation of the investigated compounds in electrospray mass spectrometry (ESI-MS) and PF-MEKC–ESI-MS analysis of fluoxymesterone, androstenedione and testosterone are described.

2. Investigated compounds

The systematic names (average) molecular masses and estimated pK_a values of the investigated compounds are presented in Table 1.

Androstenedienone (Fig. 1a) is the major immediate precursor of testosterone in the human body. Metabolism of orally administered androstenedione and urinary excretion of androstenedione and testosterone metabolites has been investigated [10]. It was concluded that the administration of androstenedione increases the excretion rate of, e.g. conjugated testosterone, and that androstenedione is largely metabolised before release into general circulation.

Fluoxymesterone (Fig. 1b) is a synthetic compound commonly used as a doping agent. Metabolism of fluoxymesterone in human has been investigated by oral administration of fluoxymesterone to a male volunteer [11]. Both free fluoxymesterone and its metabolites were derivatised and determined in urine by gas chromatography–mass spectrometry (GC–MS).

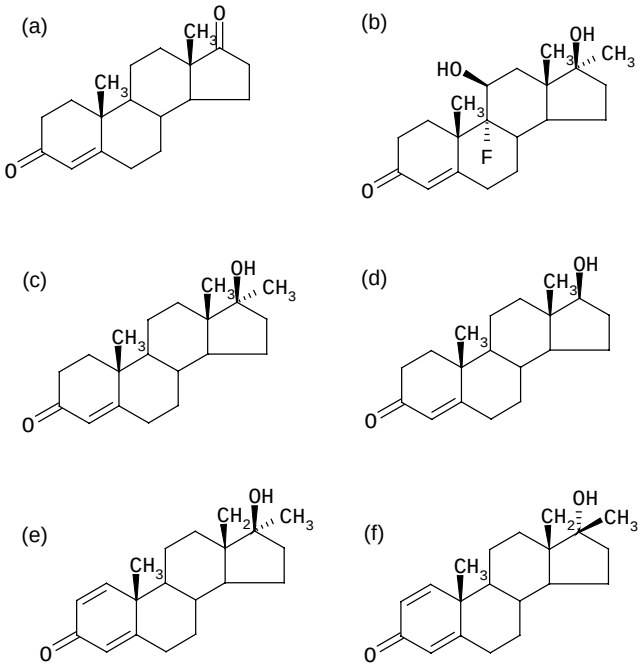

Fig. 1. Structures of investigated compounds: (a) androstenedione, (b) fluoxymesterone, (c) methyltestosterone, (d) testosterone, (e) metandienone and (f) 17-epimetandienone.

Methyltestosterone (Fig. 1c), a synthetic derivative of testosterone (Fig. 1d, Table 1), undergoes a more concise metabolism pathway than testosterone. Hence, methyltestosterone, too, is an attractive doping agent. The metabolism of testosterone and 17α-methyltestosterone in human has been investigated in a similar way to that of fluoxymesterone [11]. For the present work, methyltestosterone was selected as internal standard (I.S.), since it is a synthetic compound and normally not present in urine. Other reasons were its appropriate migration time relative to the migration times of other compounds, and the symmetry and low standard deviation of the peak.

Metandienone (Fig. 1e) is another commonly abused compound in sports. Its metabolism in human body has been widely investigated [3,11–13]. After oral administration of metandienone, two major metabolites appear in urine, namely 17-epimetandienone (Fig. 1f) and 6β-hydroxy-17-epimetandienone. These metabolites are exclusively excreted in unconjugated form [13,14]. Metabolism of metandienone after oral administration has been investigated in a similar way to the metabolism of fluoxymesterone [11].

According to literature [7,11–14], the appearance of free androstenedione, fluoxymesterone, metandienone, methyltestosterone, testosterone and 17-epimetandienone in urine is likely or possible after an oral administration.

Table 1
Investigated compounds (trivial names, systematic names, average molecular masses and estimated pK_a values)

Trivial name	Systematic name	M_r (g/mol)	pK_a
Fluoxymesterone	9α-Fluoro-11β,17β-dihydroxy-17α-methylandrost-4-en-3-one	336.45	14.37
Androstenedione	Androst-4-ene-3,17-dione	286.41	None
Metandienone	17β-Hydroxy-17α-methylandrosta-1,4-dien-3-one	300.44	16.00
Testosterone	17β-Hydroxyandrost-4-en-3-one	288.43	16.18
Methyltestosterone	17β-Hydroxy-17α-methylandrost-4-en-3-one	302.46	16.00
17-Epimetandienone	17α-Hydroxy-17β-methylandrosta-1,4-dien-3-one	300.44	16.00

The pK_a values of the functionalities were calculated with Pallas 1.2 program (CompuDrug Chemistry, Budapest, Hungary).

Determining the concentrations of these free endogenous and synthetic AAS in urine is an essential part of doping control and also of interest in medical diagnostics.

3. Analysis of anabolic steroids

Determining the low concentrations of AAS in biological samples is a challenging task. AAS are hydrophobic, non-polar compounds with high pK_a values and very similar structures. Separation of isomers is often required (e.g. metandienone and 17-epimetandienone). The low concentrations and close structural similarity of AAS demand a separation technique with high resolution and sensitive detection capability.

Confirmation and quantitation of anabolic agents and their metabolites in human urine is conventionally performed by GC–MS [6,7,12–20]. Solid-phase extraction (SPE) of urine is required before the analysis. Often enzymatic hydrolysis of the steroid conjugates followed by liquid–liquid extraction (LLE) is performed as well [13]. Since some anabolic steroids and their metabolites possess hydroxyl and carbonyl groups in their structure, derivatisation is necessary, too. With GC–MS, very low limits of detection (LODs) can be achieved for AAS. LODs down to 5 ng/L have been reported [16]. However, a serious drawback of GC–MS determinations of AAS is that sample pre-treatment is always laborious and time-consuming [13].

Liquid chromatography (LC) and capillary electrophoresis (CE) are highly promising methods for fast steroid screening of body fluids. No derivatisation is required. Several HPLC [21–23] and HPLC–MS [24–27] assays for anabolic steroids and their metabolites have been carried out. LODs obtained with HPLC for fluoxymesterone, testosterone and methyltestosterone in the range of 64–70 ng/L have been reported [22]. Recently, an on-line sample enrichment procedure based on column-switching HPLC using gradient elution technique combined with MS was described for the determination of 6β-hydroxytestosterone with the limit of quantitation (LOQ) of 20 μg/L [27]. However, sample as well as reagent consumption in HPLC is significantly greater than in CE. Mobile phases in HPLC are often flammable, expensive and toxic.

CE has been employed in anabolic steroid analysis much less frequently than HPLC. Only a few studies have focused on AAS, namely testosterone, dimethyltestosterone and testosterone propionate [28,29], and androstenedione, testosterone and 6α, 6β, 7α, 16β, 16α, 2α and 2β-hydroxytestosterone [30]. LODs for testosterone of 500 and 115 μg/L have been reported [29,30]. The lack of attention may be due to the fact that many anabolic steroids have very similar hydrophobic structures (Fig. 1), and do not possess electric charge within pH range 2–12, the range applicable for CE (Table 1). In the CE capillary, uncharged analytes migrate along the electro-osmotic flow when voltage is applied and no separation is obtained without the use of a pseudostationary phase.

The problem of uncharged analytes was first solved by Terabe et al. [31] in 1984, who introduced a micellar electrokinetic capillary chromatographic (MEKC) method. In MEKC, the analytes separate as they migrate in a capillary filled with background electrolyte (BGE) containing surfactants at concentrations higher than their critical micelle concentration (CMC). Micelles are dynamic structures generally possessing a multiple charge. The most popular surfactant in MEKC is sodium dodecyl sulphate (SDS), which forms anionic monomers. Mixed micelles consisting of SDS and anionic bile salts have been widely used, for example in assays of corticosteroids [32–34], where separation has been alleged to be impossible with the use of SDS alone [32]. As normal polarity is applied, negative micelles migrate very slowly towards the cathode, and lag the electro-osmotic flow. The separation of uncharged analytes is based on their partitioning between micelles and the BGE. To be separated, the analytes must have different micelle–water partition coefficients, P_{mw}. The separation process incorporates hydrophobic as well as other interactions [35].

In order to couple MEKC to mass spectrometry, Nelson et al. [36,37] first used PF-MEKC. Non-volatile surfactants like SDS easily contaminate the MS instrument and, in PF-MEKC, contamination can be avoided since only a part of the CE capillary is filled with micellar solution. Since uncharged analytes migrate towards the cathode faster than negatively charged micelles, the voltage can be switched off after the last analyte has entered the MS source. Before the next analysis, the capillary is rinsed to remove excess surfactant.

In PF-MEKC, the surfactant concentration and the micellar zone length are the main experimental parameters that can be varied to optimise resolution [38]. The behaviour

of the injected micellar solution in PF-MEKC differs from that in traditional MEKC. In general, micelles possess a much higher effective mobility than the individual surfactant monomers. This is due to the fact that micelle formation leads to a reduction of viscosity. In PF-MEKC, micelles that migrate into the buffer at the rear side of the surfactant zone experience an aqueous phase without surfactant monomers. The total amount of micelles gradually decreases, and finally a steady state is reached where all surfactant molecules migrate as monomers. The time required to reach a steady state is proportional to the length and surfactant concentration of the injected micellar zone [38]. Since the amount of micelles decreases with time, compounds with a higher retention factor will experience a smaller effective separation length. One obvious drawback is that efficiencies are often lower with PF-MEKC than with normal MEKC [38].

It has been suggested, that comparable migration times can be obtained by different combinations of micellar zone length and surfactant concentration [38]. However, in PF-MEKC, an additional band broadening at the micellar zone-buffer boundary occurs. This is caused by a partial laminar flow, which is produced due to the difference in the electric field strengths of the zones [38]. Since this band broadening increases with higher retention factors, a low surfactant concentration is to be preferred.

In the present study, a PF-MEKC method suitable for coupling with ESI-MS is described and applied to the identification of six anabolic steroids.

4. Experimental

4.1. Chemicals

Fluoxymesterone (4-androsten-9α-fluoro-17α-methyl-11β,17β-diol-3-one, $C_{20}H_{29}FO_3$) was purchased from Steraloids (Newport, USA). Androstenedione (androst-4-ene-3, 17-dione, $C_{19}H_{26}O_2$), metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one, $C_{20}H_{28}O_2$), methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one, $C_{20}H_{30}O_2$), 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione, $C_{21}H_{30}O_3$), DHEA (5-androstene-3β-ol-17-one, $C_{19}H_{28}O_2$) and 17-epimetandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one, $C_{20}H_{28}O_2$) were a gift from United Laboratories (Helsinki, Finland). Testosterone was a gift from the Laboratory of Organic Chemistry (University of Helsinki, Finland). Ammonium acetate was purchased from Fluka (Buchs, Switzerland). SDS (approx. 99%) was from Sigma (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. The deionised water was produced with a Milli-Q gradient A10 system (Millipore, Bedford, MA, USA).

4.2. Instruments

4.2.1. pH meter

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).

4.2.2. PF-MEKC–DAD

PF–MEKC experiments were performed using a Beckman P/ACE MDQ instrument equipped with DAD system (Beckman Instruments, Fullerton, CA, USA). The dimensions of a fused-silica capillary (Composite Metal Services, The Chase, Hallow, UK) were 50 μm i.d. × 375 μm o.d. The total length of the capillary was 80 cm and the effective length 70 cm. Before analysis, the capillary was conditioned by rinsing at 138 kPa (20.0 psi) for 15 min with 0.1 M NaOH, 10 min with deionised water and 15 min with separation buffer, respectively. The capillary was thermostated at 22 °C, whereas samples were kept at 18 °C. The MEKC runs were performed at +25 kV separation voltage (normal polarity). Before every run, the capillary was rinsed for 5 min with the separation buffer at 138 kPa. The micellar solution was injected at 3447 Pa (0.5 psi) for 99.9 s. The length of the micellar plug was 3.5 cm assuming that the viscosity of the micellar solution is equal to that of water (calculated with CE Expert 1.0 Program, Beckman Instruments). Thereafter the sample was injected to the capillary at 3447 Pa for 5 s. Before separation voltage was applied, the electrodes and capillary tips were dipped into deionised water. The analytes were detected at 247 nm wavelength, which gave good response for all analytes.

4.2.3. Direct inlet MS

The mass spectrometer was a Quattro II triple quadrupole instrument equipped with an ESI interface (Micromass, Manchester, UK). Ionisation of the investigated compounds was studied in positive ionisation mode with a scan range of m/z 50–800 in the MS mode and 20 ($[M+H]^+ + 20$) in the MS–MS mode. The samples were introduced with a 100 μl syringe (Hamilton, Reno, NV, USA) with the help of a microsyringe pump (Harvard Apparatus, South Natick, MA, USA) operating at a flow rate of 5 μL/min. The flow rate of the drying gas (N_2) was 250 L/h, and that of the nebulising gas (N_2) 10–20 L/h. The capillary, high-voltage (HV) lens and cone voltages were +4.0 kV, +0.5 kV and +40 V, respectively, and the source temperature was +70 °C. In the MS–MS mode, the applied collision energy was 20–30 eV and the pressure of collision gas (Ar) 1.7 μbar. The scan time was 3.1 s and the interscan delay 0.14 s.

4.2.4. PF-MEKC–ESI-MS

PF-MEKC–ESI-MS experiments were performed using the CE and ESI-MS instruments described above.

The length of the capillary was 80 cm. The instruments were connected through a commercial interface (Micromass) based on a coaxial sheath flow. The sheath liquid was introduced with a 1 mL syringe with a flow rate of 10 μL/min. In the PF-MEKC-MS analysis, the MEKC conditions were as described in Section 4.2.2 with the following modifications. The separation was performed at +20 kV and 3447 Pa (0.5 psi) back pressure. Thermosetting the capillary was not possible in PF-MEKC–ESI-MS. In PF-MEKC–ESI-MS analyses, the HV lenses were removed from the ionisation chamber. It is advised by the manufacturer to set the potential of the HV lenses to zero when coupling CE with ESI-MS. However, we have earlier found that a small potential is nonetheless induced impairing the signal [39]. Several different capillary and cone voltages were tested, and they were finally set at +3.5 kV and +40 V. The source temperature was 70 °C. The flow rate of the nebulising gas was 50 L/h. No drying gas was used. Prior to analysis, the CE–ESI-MS system was tuned with a 10 mg/L testosterone solution introduced through the CE capillary at 6895 Pa (1.0 psi) and with a sheath liquid flow of 10 μL/min. For the selected ion monitoring (SIM) analysis, base peaks (100%) were selected for each of the compounds (fluoxymesterone, androstenedione and testosterone). The ions were monitored with a dwell time of 0.08 s and an interchannel delay of 0.00 s.

4.3. Preparation of samples and solutions

The stock solutions of 1 mg/mL of fluoxymesterone, testosterone, androstenedione and metandienone, 0.1 mg/mL of methyltestosterone and 0.5–1 mg/mL (exact concentration not known) of 17-epimetandienone were prepared in ethanol and stored at −20 °C in 1.5 mL glass vessels. The final solutions used in the analysis were made by diluting the stock solutions in a mixture of methanol–20 mM ammonium acetate (pH 9.5) (50:50, v/v). The electrolyte solution was 20 mM ammonium acetate (pH 9.5). The pH was adjusted with 0.1 M ammonium hydroxide. The electrolyte solution was filtered with a 0.45 μm MF filter (Millipore, Ireland) and stored at +4 °C in a glass vessel. The stock solution of 100 mM SDS in 20 mM ammonium acetate, pH 9.0, was stored at room temperature in a glass container, and not filtered before use. The stock solution of 36.1 mM sodium taurocholate in 20 mM ammonium acetate (pH 9.0) 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, 1.1 mM sodium taurocholate and 6.7% methanol in 18.7 mM ammonium acetate, pH 9.5. All solutions were sonicated for 15 min at room temperature before MEKC runs. The sheath liquid in PF-MEKC–ESI-MS was a mixture of methanol–20 mM ammonium acetate, pH 9.5 (50:50, v/v) containing 0.1% acetic acid.

4.4. 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 in the range of 0.1–5 mg/L of fluoxymesterone, androstenedione, testosterone and metandienone, 0.05–2.5 mg/L of methyltestosterone and between 0.1–0.2 and 5.0–10.0 mg/L of 17-epimetandienone. Each sample was injected eight times ($n = 8$).

5. Results and discussion

5.1. Optimisation of PF-MEKC conditions

Ammonium acetate (20 mM, pH 9.5) was selected as the electrolyte salt, because it is suitable for MS coupling. It also enabled a relatively strong electro-osmotic flow in the capillary. Anionic surfactants were chosen as pseudostationary phase, because anionic micelles and surfactant monomers migrate to the detector slower than neutral analytes when normal polarity is applied for the separation. Thus voltage can be switched off when the last analyte has entered the ESI-MS detector. Another advantage of anionic surfactants is that the ions are repulsed by the negatively charged silanol groups of the silica capillary, and do not significantly adsorb on the capillary wall.

The optimisation of the surfactant concentration is important in MEKC. Critical micelle concentration must be exceeded for retention of analytes in the micellar phase. In 20 mM ammonium acetate (pH 9.5), the CMC value of SDS is 3.4–3.5 mM [40]. In the present study, a relatively low surfactant concentration was preferred to decrease band broadening at the micellar zone-buffer boundary. Additionally, a hydrodynamically injected, long micellar zone was preferred to decrease micellar zone degradation.

According to literature [32], it was assumed that the separation of the anabolic steroids would not be possible with use of SDS alone. However, separation of 10 corticosteroids in 25 mM SDS–25 mM borate buffer (pH 9.5) has been reported [41], but this electrolyte is inappropriate for ESI-MS detection because of non-volatile buffer ions. In the present study, separation of the investigated AAS was tested with several different SDS concentrations (25–50 mM) and micellar solution injection times (8–99.9 s). SDS proved to be inadequate (electrolyte solution and run conditions as described in Section 4, data not shown). However, a mixed micellar solution consisting of SDS and sodium taurocholate improved the separation. Several different micellar solution compositions and injection times (80–120 s) were tested. The chemical optimisation of the micellar solution resulted in a mixture consisting of 29.3 mM SDS, 1.1 mM sodium taurocholate and 6.7% methanol in 18.7 mM ammonium acetate at pH 9.5 (injection time 99.9 s). The solution provided baseline separation of the six anabolic steroids in less than 14 min with the following migration

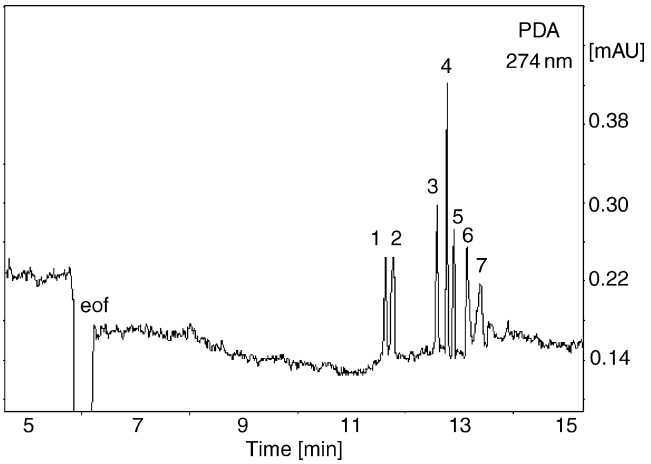

Fig. 2. Separation of anabolic androgenic steroids with PF-MEKC. Peak identification: (1) fluoxymesterone, (2) androstenedione, (3) metandienone, (4) testosterone, (5) methyltestosterone, (6) 17-epimetandienone (7) taurocholate. Eof: electro-osmotic flow. Conditions: buffer 20 mM ammonium acetate (pH 9.5), micellar solution: 29.3 mM SDS, 1.1 mM sodium taurocholate and 6.7% methanol in 18.7 mM ammonium acetate (pH 9.5), capillary 80 cm (70 cm to detector), + 25 kV, 22 °C (capillary), 18 °C (samples), detection at 247 nm, injection 99.9 s at 3.4 kPa (micellar solution) and 5 s at 3.4 kPa (sample). Concentrations: 2 mg/L of fluoxymesterone, androstenedione, metandienone and testosterone, 1 mg/L of methyltestosterone and 2–4 mg/L of 17-epimetandienone.

order: fluoxymesterone, androstenedione, metandienone, testosterone, methyltestosterone and 17-epimetandienone (Fig. 2). To ensure that sodium taurocholate really affected on the separation, the same experiment was performed with the exception that sodium taurocholate was replaced with the electrolyte. Bare SDS indeed proved to be insufficient (data not shown).

In MEKC, the separation of electrically neutral compounds is based on differences in their hydrophobicity (P_{mw}) and interactions between surfactants and analytes. In the present work, fluoxymesterone, having the greatest molecular mass but the lowest hydrophobicity of the analytes, migrates most rapidly. Metandienone and 17-epimetandienone, molecular isomers having the same molecular mass, clearly act differently in the micellar zone. 17-Epimetandienone only just separates from the micellar phase, whereas metandienone is detected almost 1 min before taurocholate. Under the same conditions, detection of DHEA (5-androstene-3β-ol-17-one) was not possible, and 17α-hydroxyprogesterone migrated at the same velocity as methyltestosterone (data not shown). Methanol, which is not retained by the micellar phase, was a marker for the electro-osmosis. Adding methanol to the micellar solution also slightly improved the profiles of the sample zones.

5.2. Choice of the analyte solution composition

Various sample solvent compositions were tested to optimise the PF-MEKC analyses of the steroids. At first, bare ethanol and methanol were used to ensure dissolution of the hydrophobic analytes, but they were later rejected because of significant evaporation (causing poor reproducibility). A mixture of 15 mM SDS in 20 mM ammonium acetate (pH 9.0)–methanol (20:80, v/v) gave poor reproducibility as well. Finally, dissolving the analytes in a methanol–20 mM ammonium acetate (pH 9.5) (50:50, v/v) mixture provided good separation and reproducibility.

5.3. PF-MEKC validation data

The PF-MEKC results obtained with the validated system are given in Table 2. The LOD for every steroid was determined as a signal-to-noise (S/N) ratio of 3. The obtained LOD value for testosterone (40 μg/L) is lower compared to that earlier obtained with MEKC (115 μg/L, S/N = 3) [30]. However, the LOD values obtained for fluoxymesterone (45 μg/L), testosterone and methyltestosterone (59 μg/L) are significantly higher compared to those obtained with HPLC (64, 67 and 70 ng/L, respectively) [22]. It is noteworthy, however, that sample consumption in the present study (3.5 nL, calculated with CE Expert 1.0 Program, Beckman Instruments) was remarkably lower compared to that in HPLC (20 μL) [22]. Also with GC–MS, lower detection limits (5 ng/L) than the ones obtained here for AAS have been reported [16]. However, GC–MS analyses always require derivatisation, which was avoided here.

The linearity of the compounds was good in the range tested (0.1–5.0 mg/L). The linearity coefficients (r^2) ranged from 0.994 (fluoxymesterone) to 0.999 (testosterone, metandienone and androstenedione). The repeatabilities of the compound peak areas varied between 11.22 (testosterone) and 15.83% R.S.D. (metandienone), and the repeatabilities of the migration times between 0.76 and 0.81% R.S.D. Separation of the steroid analytes is shown in Fig. 2. Baseline separation is attained in less than 14 min with the following migration order: fluoxymesterone, androstenedione,

Table 2
PF-MEKC validation data of the anabolic androgenic steroids ($n = 8$)

	Fluoxymesterone	Androstenedione	Metandienone	Testosterone	Methyltestosterone (I.S.)	17-Epimetandienone
LOD (μg/L)	45	39	90	40	59	45–90
R.S.D. (%) (peak area)	14	12	16	11	15	13
R.S.D. (%) (migration time)	0.80	0.79	0.81	0.80	0.80	0.76
Linearity (r^2)	0.994	0.999	0.999	0.999	–	0.998

Table 3
Ionisation of the investigated compounds in positive ion mode (direct inlet MS)

	Fluoxymesterone	Androstenedione	Metandienone	Testosterone	Methyltestosterone (I.S.)	17-Epimetandienone
Most intense ion (100%) in positive ion MS mode	$[M+H]^+$ at *m/z* 337	$[M+H]^+$ at *m/z* 287	$[M+H]^+$ at *m/z* 301	$[M+H]^+$ at *m/z* 289	$[M+H]^+$ at *m/z* 303	$[M-H_2O+H]^+$ at 283
Second most intense ion in positive ion MS mode	$[M+Na]^+$ at *m/z* 359 (6.3%)	$[M+Na]^+$ at *m/z* 309 (17.5%)	$[M+Na]^+$ at *m/z* 323 (23.8%) $[M-H_2O+H]^+$ at 283 (36.3%)	$[M+Na]^+$ at *m/z* 311 (19.0%)	$[M+Na]^+$ at *m/z* 325 (17.7%)	$[M+H]^+$ at *m/z* 301 (73.1%) $[M+Na]^+$ at *m/z* 323 (74.6%)
Most intense daughter ions in the MS–MS mode	None	*m/z* 97	*m/z* 121	*m/z* 97	*m/z* 97	*m/z* 121
		m/z 109	*m/z* 149	*m/z* 109	*m/z* 109	*m/z* 149

Conditions presented in Section 4. In the MS–MS mode, the $[M+H]^+$ ions were fragmented using a collision energy of 20 eV (and 30 eV for fluoxymesterone).

metandienone, testosterone, methyltestosterone (I.S.) and 17-epimetandienone.

5.4. Ionisation of the investigated compounds

Ionisation of the investigated compounds was studied in positive ion mode. The concentrations of the standards were 10 mg/L of fluoxymesterone, androstenedione, metandienone and testosterone, 5 mg/L of methyltestosterone and 5–10 mg/L of 17-epimetandienone. The results in Table 3 show that in the MS mode, the most intense signal was typically the protonated molecular ion $[M+H]^+$. In addition, sodium adducts $[M+Na]^+$ were detected. The ratio of $[M+H]^+$ and $[M+Na]^+$ was over 4.2 except for 17-epimetandienone, which gave its strongest signal at *m/z* 283 corresponding to $[M-H_2O+H]^+$. The ratio of $[M+H]^+$ and $[M+Na]^+$ for 17-epimetandienone was close to 1.0. For every investigated analyte, also dimeric ions $[2M+H]^+$ and $[2M+Na]^+$ were detected. However, these signals were weak compared to those for $[M+H]^+$ and $[M+Na]^+$. In the MS–MS mode, the $[M+H]^+$ ions were fragmented using a collision energy of +20 eV (and +30 eV for fluoxymesterone). For androstenedione, testosterone and methyltestosterone, the most intense daughter ions were detected at *m/z* 97 and 109. Metandienone and 17-epimetandienone produced fragment ions of *m/z* 121 and 149, but fluoxymesterone did not produce any fragment ions. It is likely that the electronegative fluorine atom in fluoxymesterone protects it from fragmentation.

5.5. PF-MEKC–MS analysis of fluoxymesterone, androstenedione and testosterone

Separation and identification of fluoxymesterone, androstenedione and testosterone by PF-MEKC–ESI-MS was studied. The standard contained 10 mg/L of each analyte in methanol–20 mM ammonium acetate (pH 9.5) (50:50, v/v). The PF-MEKC method was easily connected to ESI-MS with only a few adjustments: The separation was performed at +20 kV using 3447 Pa (0.5 psi) back pressure. Thermosetting the capillary was not possible in PF-MEKC–ESI-MS. With the HV lenses removed, the use of drying gas could totally be avoided, and signal intensities were strengthened.

The analysis of fluoxymesterone, androstenedione and testosterone (10 mg/L of each) by PF-MEKC–ESI-MS is presented in Fig. 3. The ESI-MS detector seems to be very selective. Only the desired ions are detected on the selected channels (data collection in SIM mode).

Identification of the analytes with PF-MEKC–ESI-MS is performed about two min later than with PF-MEKC using DAD. Fluoxymesterone, androstenedione and testosterone can be identified with PF-MEKC–ESI-MS in less than 16 min.

Separation of the analytes is successful even when the capillary is coupled to the ESI-MS interface. This provides

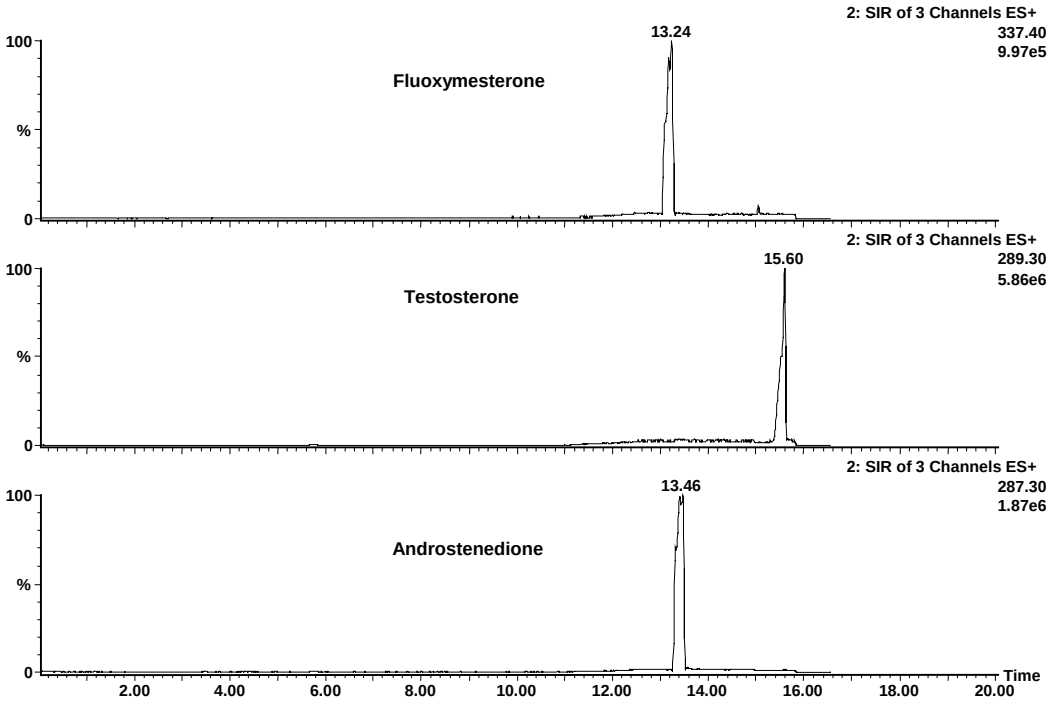

Fig. 3. Identification of fluoxymesterone, testosterone and androstenedione with PF-MEKC–ESI-MS. Sample concentration 10 mg/L. Optimized conditions in Section 4. *x*-axis: migration time in minutes. *y*-axis: normalised detector response to 100%. Selected ion monitoring (SIM): fluoxymesterone *m*/*z* 337.40, testosterone *m*/*z* 289.30 and androstenedione *m*/*z* 287.30.

many advantages. The time for the analyte to be detected in the MS detector is proportional to the time the analyte spends in the silica capillary partially filled with the micellar solution. Thus the detection time of each analyte can be predicted. Without PF-MEKC separation and a predictable detection time, identification of fluoxymesterone would only be based on the ion $[M + H]^+$ produced in positive ion MS mode, since no fragment ions can be produced in the MS–MS mode (Table 3). Additionally, when identification of isomers, e.g. metandienone and 17-epimetandienone, is desired, separation of the compounds would be helpful, since they produce similar ionisation patterns in positive ion MS and MS/MS modes (Table 3).

6. Concluding remarks

The separation of six anabolic androgenic steroids (fluoxymesterone, androstenedione, methyltestosterone, testosterone, metandienone and 17-epimetandienone) by PF-MEKC and their monitoring with a photodiode array detector was validated. The method utilises a mixed micellar solution consisting of SDS and sodium taurocholate. Baseline separation of the analytes was achieved in less than 14 min. Limits of detection were lower than the ones reported earlier with MEKC, and the linearity of the compounds was good in the range tested. Undoubtedly, however, sample concentration must be performed when real biological sample analysis is desired. The separation method was designed to be easy to couple with ESI-MS. Indeed, a successful PF-MEKC–ESI-MS assay for fluoxymesterone, androstenedione and testosterone was performed.

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