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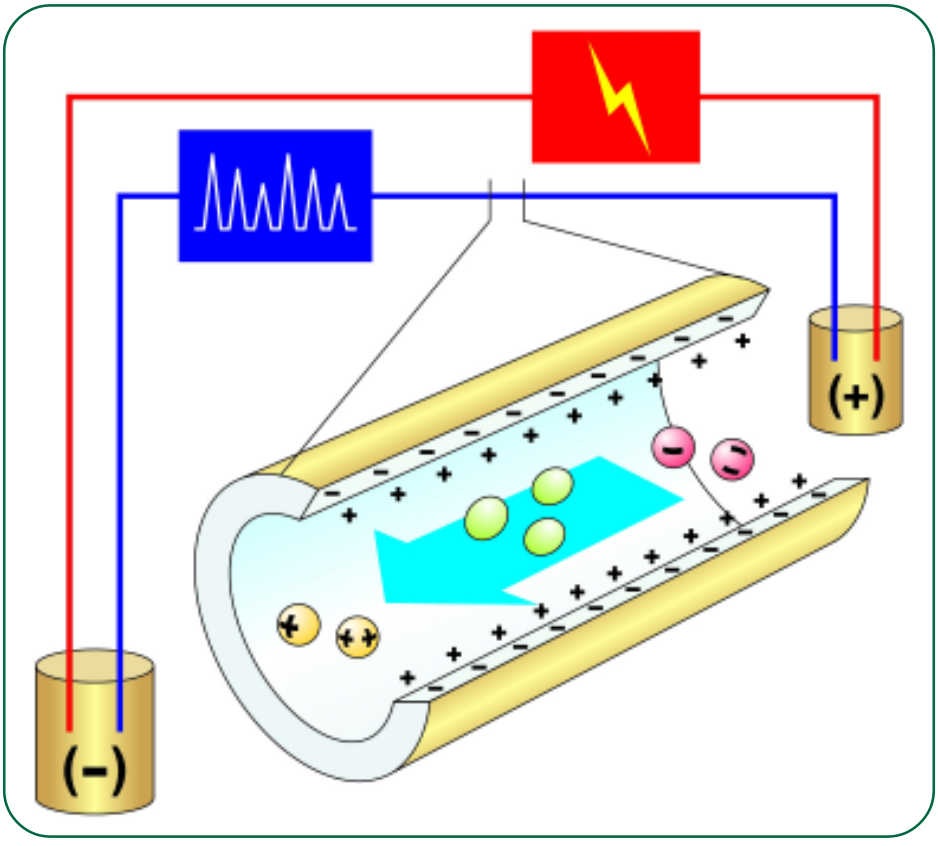

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Use of non-specific and specific interactions in the analysis of testosterone and related compounds by capillary electromigration techniques

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Amundsen, Lotta K. Use of non-specific and specific interactions in the analysis of testosterone and related compounds by capillary electromigration techniques [Epäspesifisten ja spesifisten vuorovaikutuksien hyödyntäminen testosteronin ja sen sukuisten steroidien kapillaarielektromigraatioanalyyseissä]. Espoo 2008. VTT Publications 675. 109 p. + app. 56 p.

Keywords capillary electromigration techniques, capillary electrophoresis, micellar electrokinetic chromatography, electrospray ionization mass spectrometry, immunoaffinity, solid-phase extraction, Fab fragment, testosterone, epitestosterone, androgen, anabolic steroid, albumin

Abstract

Determination of testosterone and related compounds in body fluids is of utmost importance in doping control and the diagnosis of many diseases. Capillary electromigration techniques are a relatively new approach for steroid research. Owing to their electrical neutrality, however, separation of steroids by capillary electromigration techniques requires the use of charged electrolyte additives that interact with the steroids either specifically or non-specifically. The analysis of testosterone and related steroids by non-specific micellar electrokinetic chromatography (MEKC) was investigated in this study. The partial filling (PF) technique was employed, being suitable for detection by both ultraviolet spectrophotometry (UV) and electrospray ionization mass spectrometry (ESI-MS). Efficient, quantitative PF-MEKC–UV methods for steroid standards were developed through the use of optimized pseudostationary phases comprising surfactants and cyclodextrins. PF-MEKC–UV proved to be a more sensitive, efficient and repeatable method for the steroids than PF-MEKC–ESI-MS. We discovered that in PF-MEKC analyses of electrically neutral steroids, ESI-MS interfacing sets significant limitations not only on the chemistry affecting the ionization and detection processes, but also on the separation. The new PF-MEKC–UV method was successfully employed in the determination of testosterone in male urine samples after microscale immunoaffinity solid-phase extraction (IA-SPE). The IA-SPE method, relying on specific interactions between testosterone and a recombinant anti-testosterone Fab fragment, is the first such method described for testosterone. Finally, new data for interactions between steroids and human and bovine serum albumins were obtained through the use of affinity capillary electrophoresis. A new algorithm for the calculation of association constants between proteins and neutral ligands is introduced.

Amundsen, Lotta K. Use of non-specific and specific interactions in the analysis of testosterone and related compounds by capillary electromigration techniques [Epäspesifisten ja spesifisten vuorovaikutuksien hyödyntäminen testosteronin ja sen sukuisten steroidien kapillaarielektromigraatioanalyyseissä]. Espoo 2008. VTT Publications 675. 109 s. + liitt. 56 s.

Avainsanat capillary electromigration techniques, capillary electrophoresis, micellar electrokinetic chromatography, electrospray ionization mass spectrometry, immunoaffinity, solid-phase extraction, Fab fragment, testosterone, epitestosterone, androgen, anabolic steroid, albumin

Tiivistelmä

Testosteronin ja sen sukuisten steroidien määrittäminen kehon nesteistä on keskeistä doping-valvonnassa sekä monien sairauksien diagnosoinnissa. Kapillaarielektromigraatiotekniikat ovat varsin uusi lähtökohta steroiditutkimuksille. Steroidien sähköisestä varauksettomuudesta johtuen kapillaarielektromigraatiotekniikoiden soveltaminen steroidien erottamiseen edellyttää ionisoituvien orgaanisten lisäaineiden käyttöä elektrolyyttiliuoksessa. Lisäaineilla voi olla joko spesifisiä tai epäspesifisia vuorovaikutuksia steroidien kanssa. Tässä tutkimuksessa testosteronin ja sen sukuisten steroidien määrittämistä tutkittiin epäspesifisellä misellisellä sähkökineettisellä kromatografialla (MEKC). Osittaistäyttötekniikan (PF) ansiosta steroidit voitiin tunnistaa sekä ultraviolettispektrofotometrisesti (UV) että sähkösumutus-ionisaatiomassaspektrofotometrisesti (ESI-MS). Steroidistandardeille kehitettiin tehokkaat ja kvantitatiiviset PF-MEKC–UV-menetelmät käyttäen lisäaineina tensidejä sekä syklodekstriinejä. PF-MEKC–UV osoittautui herkemmäksi, tehokkaammaksi ja toistettavammaksi menetelmäksi kuin PF-MEKC–ESI-MS. Tutkimuksessa havaittiin, että ESI-MS-liitäntä asettaa huomattavia kemiallisia rajoituksia paitsi ionisaatio- ja detektointitapahtumille myös erotustapahtumalle. Uutta PF-MEKC–UV-menetelmää voitiin käyttää testosteronin määrittämiseen miesten virtsanäytteistä miniatyrisoidun immunoaffiniteettikiinteäfaasiuuton (IA-SPE) jälkeen. Kyseinen IA-SPE perustuu vuorovaikutuksiin testosteronin ja sille spesifisen rekombinantin Fab-fragmentin välillä. Aikaisemmin IA-SPE:tä ei ole sovellettu testosteronin uuttamiseen. Lopuksi, uutta tietoa steroidien sekä ihmisen ja naudan seerumin albumiinin välisistä vuorovaikutuksista saatiin affiniteettikapillaarielektroforeesin avulla. Tutkimuksessa kehitettiin uusi algoritmi, jonka avulla voidaan laskea proteiinien ja neutraalien ligandien välisiä sitoutumisvakioita.

To my daughter Ada Solveig Helena

Preface

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All supervisors and students in ISB contributed through our fruitful and enthusiastic annual meetings.

Dr. Kathleen Ahonen revised the language of the articles I–III and V, and the thesis summary in professional fashion.

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Finally, I thank my husband Jarle for his love and support. I feel privileged to share each day of my life with you. You and our little daughter Ada are the most precious things in my life. I love you two so much.

In Espoo, April 2008

Lotta

List of publications

This thesis is based on the following publications, hereafter referred to by their Roman numerals (I–V):

I Amundsen, L. K., Kokkonen, J. T., Rovio, S. and Sirén, H. Analysis of anabolic steroids by partial filling micellar electrokinetic capillary chromatography and electrospray mass spectrometry. *Journal of Chromatography A* 2004, *1040*, 123–131.

II Amundsen, L. K. and Sirén, H. Partial filling micellar electrokinetic chromatography analysis of androgens and testosterone derivatives using two sequential pseudostationary phases. *Journal of Chromatography A* 2006, *1131*, 267–274.

III Amundsen, L. K., Nevanen, T. K., Takkinen, K., Rovio, S. and Sirén, H. Microscale immunoaffinity SPE and MEKC in fast determination of testosterone in male urine. *Electrophoresis* 2007, *28*, 3232–3241.

IV Amundsen, L. K., Kokkonen, J. T. and Sirén, H. Comparison of partial filling MEKC analyses of steroids with use of ESI-MS and UV spectrophotometry. *Journal of Separation Science* 2008, *31*, 803–813.

V Amundsen, L. K. and Sirén, H. Determination of association constants between steroid compounds and albumins by partial filling ACE. *Electrophoresis* 2007, *28*, 3737–3744.

Some additional, unpublished data is included.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp)

List of abbreviations

A	Androstenedione
AAS	Anabolic androgenic steroid
ACE	Affinity capillary electrophoresis
ACN	Acetonitrile
APCE	Affinity probe capillary electrophoresis
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BGE	Background electrolyte
BSA	Bovine serum albumin
CD	Cyclodextrin
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CMC	Critical micelle concentration
CZE	Capillary zone electrophoresis
DHEA	Dehydroepiandrosterone
E	Epitestosterone
E. coli	*Escherichia coli*
EKC	Electrokinetic chromatography
ELISA	Enzyme-linked immunosorbent assay
EOF	Electroosmotic flow
ESI-MS	Electrospray ionization mass spectrometry
F	Fluoxymesterone
GC–MS	Gas chromatography–mass spectrometry
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
Hp	*Helix pomatia*
HPLC	High performance liquid chromatography
HSA	Human serum albumin

IACE	Immunoaffinity capillary electrophoresis
IA-SPE	Immunoaffinity solid-phase extraction
i.d.	Inner diameter
IDA	Iminodiacetic acid / iminodiacetate
IS	Immunosorbent
i.s.	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC–MS	Liquid chromatography–mass spectrometry
LIF	Laser induced fluorescence
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
MT	Methyltestosterone
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PF-ACE	Partial filling affinity capillary electrophoresis
PF-MEKC	Partial filling micellar electrokinetic chromatography
PTFE	Polytetrafluoroethylene
SC	Sodium cholate
SDS	Sodium dodecyl sulfate
SEF_{height}	Sensitivity enhancement factor obtained from peak height
SHBG	Sex hormone binding globulin
S/N	Signal-to-noise ratio
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
T	Testosterone
UV	Ultraviolet spectrophotometry

List of symbols

C_{inj}	concentration of analyte in the injected sample zone
C_{sweep}	concentration of analyte after sweeping
ε_0	permittivity of vacuum
ε_r	relative permittivity of the electrolyte solution
E	electric field
κ^{-1}	Debye length
k	retention factor
k_{MEKC}	mass distribution ratio in micellar electrokinetic chromatography
K	distribution constant in micellar electrokinetic chromatography
K_b	association constant
K_d	dissociation constant
α	selectivity
L_{det}	effective length of the capillary
L_{packed}	length of the packed capillary in capillary electrochromatography
L_{tot}	total length of the capillary
n	molar amount of protein in PF-ACE
n_{mc}	molar amount of the analyte in the micellar phase
n_{aq}	molar amount of the analyte in the aqueous phase
μ_o	electroosmotic mobility
μ_{tot}	total mobility of an analyte
η	viscosity
pI	isoelectric point
r	inner radius of the capillary
R	resolution
v_o	linear velocity of EOF
V	voltage
V_{mc}	volume of micellar phase

V_{aq}	volume of aqueous phase
t_a	migration time of an analyte
t_{eof}	migration time of a neutral EOF marker
$t_{0,eof}$	migration time of a neutral EOF marker in the absence of protein in PF-ACE
σ	peak width (at base)
ζ	zeta potential

1. Introduction

The first capillary electromigration technique, capillary electrophoresis (CE), was introduced by Jorgenson and Lukacs in 1981 [1], and quickly became established as a tool for clinical, pharmaceutical, and bioanalytical studies. Today it is in routine use in laboratories worldwide. CE is an efficient technique for separating compounds according to their differences in charge, mass, and three-dimensional structure. The separation is typically performed in a narrow (i.d. 20–100 μm) silica capillary [2], filled with an electrolyte solution. Separation occurs when an electric field is applied across the capillary. Innumerable ionized compounds have been separated by CE, ranging from small inorganic ions to proteins and other large biomolecules.

Electrically neutral compounds, including many steroid hormones, cannot be separated in an electric field unless they are interacting with charged electrolyte additives. Thus, CE is not a suitable separation technique for them. A common capillary electromigration technique for neutral analytes is micellar electrokinetic chromatography (MEKC), introduced by Terabe et al. in 1984 [3]. In MEKC, a surfactant, such as sodium dodecyl sulfate (SDS), is added to an aqueous electrolyte solution at concentrations higher than the critical micelle concentration (CMC). At such concentrations, and at temperatures higher than the Kraft point, the surfactants begin to form dynamic micelles, and when an electric field is applied the analytes are separated according to differences in their distribution between the highly charged micelles and the aqueous electrolyte solution.

Besides their lack of charge, another challenge in the separation of steroids is their structural similarity. Related compounds often cannot be separated with a simple pseudostationary phase consisting of just one type of surfactant, such as SDS. Careful optimization of the separation process is required. Selectivity and resolution in MEKC can often be altered through use of mixed micellar solutions (comprising two or more types of surfactants) or additives, such as organic solvents and cyclodextrins.

Reliable and fast determination of the principal androgen, testosterone, and related electrically neutral steroids in body fluids is of great importance in clinical laboratories and doping control. In the diagnosis of hypogonadism, polycystic ovary disease, and growth retardation [4], for instance, their

quantification in body fluids is of utmost importance. Most traditional and routine methods for the determination of testosterone and related steroids have demonstrated pitfalls. Problems are typically associated with the complexity of sample pretreatment or insufficient specificity. Despite the challenges due to neutrality and structural similarity, the use of capillary electromigration techniques in steroid determinations is highly attractive. Relative to many conventional methods, they have considerable advantages, as will be discussed below.

In the first part of this study (I–IV), efficient MEKC methods were developed for the separation of testosterone and related steroids. The compositions of the pseudostationary phases were carefully optimized. Through use of the partial filling technique (PF), the methods were designed to allow ultraviolet (UV) and/or electrospray ionization mass spectrometric (ESI-MS) detection.

Further, the suitability of the new PF-MEKC methods for determination of testosterone in male urine samples was investigated (III). The sensitivity and specificity of the analysis were enhanced by immunoaffinity solid-phase extraction (IA-SPE), through use of a recombinant anti-testosterone antibody Fab fragment. Both in-capillary and off-line IA-SPE of testosterone were investigated. The in-capillary analyses were an application of immunoaffinity capillary electrophoresis (IACE), a subtechnique of affinity capillary electrophoresis (ACE). ACE covers all those capillary electromigration techniques in which specific, often biospecific, interactions between electrolyte additives and analytes are employed [2]. Highly specific interactions between testosterone and the anti-testosterone Fab fragment were utilized in this part of the work (III).

Finally (V), interactions between testosterone-related steroids and human and bovine serum albumins were investigated by partial filling affinity capillary electrophoresis (PF-ACE). The physiological role of some of the steroids, especially epitestosterone, has not yet been fully resolved, and the association constants for the binding of the steroids with albumins provide important new information for endocrinologists.

2. Review of the literature

The literature review begins with a look at the theory behind the capillary electromigration techniques employed in this study. The analytes of interest, testosterone and its related compounds, are introduced, and traditional methods for their determination are reviewed, along with capillary electromigration techniques currently available for steroid research. Conventional non-specific sample pretreatment methods for steroid analysis are noted and, in particular, the use of antibodies (and antibody fragments) in analyte-specific solid-phase extraction is discussed. Finally, steroid identification by electrospray ionization mass spectrometry is described.

2.1 Capillary electromigration techniques

According to IUPAC (International Union of Pure and Applied Chemistry) [2], capillary electromigration techniques are divided as follows: capillary electrophoresis (also called capillary zone electrophoresis), capillary sieving electrophoresis, capillary gel electrophoresis, capillary isotachophoresis, capillary isoelectric focusing, electrokinetic chromatography, micellar electrokinetic chromatography, microemulsion electrokinetic chromatography, affinity capillary electrophoresis, and capillary electrochromatography. The four capillary electromigration techniques employed in this work are reviewed below.

2.1.1 Capillary electrophoresis

Separations in capillary electrophoresis (CE, CZE) are usually performed in a fused silica capillary. The inner wall of the capillary consists of silanol (Si-OH) groups, which become ionized in contact with a solution of pH above 2 [2, 5]. The negatively charged silanol groups give rise to an ion layer next to the wall, in which the population of positively charged counterions predominates [2]. The cation layer consists of a stable Stern layer and a diffuse layer. When an electric field is applied across the capillary, cations present in the diffuse layer start migrating toward the cathode, dragging the surrounding solvent molecules along [6]. The bulk movement of the solution relative to the stationary charged surface is referred to as electroosmotic flow (EOF) [6]. At distances greater than that of

the width of the Stern layer, where the electroosmotic flow is zero, the EOF increases rapidly towards the center of the capillary until, at distances greater than the thickness of the diffuse layer, it becomes essentially constant [2]. In CE, the diffuse layer is very thin (on the order of the Debye length, κ^{-1}) [6] relative to the inner radius of the capillary. Thus, the EOF has a flat profile which, from the point of view of separation, is a notable advantage in comparison with the parabolic flow profiles occurring in pressure-driven techniques.

The strength of the EOF is directly proportional to the zeta potential occurring between the Stern layer and the diffuse layer [6]. The pH value of the electrolyte solution determines the degree of dissociation of the silanol groups, which in turn affects the formation of the electric double layer. Thus, high alkality results in strong EOF [5]. The electroosmotic velocity, ν_0, is expressed as [2]

$$\upsilon_0 = E\mu_0 \tag{1}$$

where E is the electric field.

In capillary electromigration this velocity is positive, by convention, in the direction from the sample introduction end to the detector end. In the reverse direction it is negative [2].

The electroosmotic mobility, μ_0, is expressed as [2]

$$\mu_0 = \frac{-(\varepsilon_r \varepsilon_0 \zeta)}{\eta} \tag{2}$$

where ε_r is the relative permittivity of the electrolyte solution (usually taken as that of the pure solvent), ε_0 is the permittivity of vacuum, ζ is the zeta potential, and η the dynamic viscosity of the electrolyte solution (where the ratio of permittivity to viscosity is assumed to be independent of the electric field of the double layer).

The dimension of μ_0 is m^2/Vs. The apparent μ_0 is often experimentally determined by

$$\mu_0 = \frac{L_{\text{det}} L_{tot}}{V t_{eof}} \tag{3}$$

where L_{det} is the the effective length of the capillary, L_{tot} the total length of the capillary, V the applied voltage, and t_{eof} the migration time of a neutral EOF marker.

EOF is often also employed to force neutral and anionic analytes toward the cathode. In CE, neutral analytes migrate along with the EOF, whereas anions, having absolute electrophoretic mobilities toward the anode, migrate more slowly. The total mobility of an analyte (a) is determined as [7]

$$\mu_{tot} = \frac{L_{\det} L_{tot}}{V t_a} \tag{4}$$

where t_a is the migration time of the analyte.

The total velocity, ν_{tot}, of an analyte is determined by its electrophoretic velocity, ν_{ep}, and EOF [2]:

$$\nu_{tot} = \nu_{ep} + \nu_0 \tag{5}$$

Approximate resolution (R) between two adjacent analyte zones (i and j) can be expressed by [8]

$$R_{ji} = \frac{t_{a,j} - t_{a,i}}{\sigma_i} \tag{6}$$

where t_a is the migration time of the analyte and σ the peak width (at base).

The selectivity (α) between two adjacent analyte zones (i and j) is determined by

$$\alpha = \frac{t_{a,j}}{t_{a,i}} \tag{7}$$

Identification of the analytes is typically performed close to the outlet end of the capillary (*e.g.* ultraviolet (UV) and laser induced fluorescence (LIF) detection), making the effective length of the capillary shorter than the total length. One important exception to this is mass spectrometric (MS) detection, in which the analytes are identified immediately after they exit the capillary.

CE is a suitable separation technique for a variety of analytes, ranging from small inorganic ions to large polymers and biomolecules. The only requirements for analytes are that they are soluble in appropriate solvents and, to be separated, they have to possess an electric charge.

CE is not an effective separation technique for testosterone and its related compounds, which are electrically neutral under pH 14–16 [9, 10]. Only a few capillary electromigration techniques provide resolution for neutral analytes. All have something in common: namely, they simultaneously combine the separation power of CE with non-specific or specific interactions between the analytes and charged electrolyte additives (mobile or immobilized) that hinder the migration of the neutral analytes with the electroosmotic flow.

2.1.2 Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (MEKC) was introduced in 1984 by Terabe et al. [3] to solve the problem of separating neutral analytes in an electric field. MEKC relies on the use of surfactants which, above the critical micelle concentration (CMC) and the Kraft point (temperature at which the solubility of the surfactant is equal to its CMC [11]), aggregate to form dynamic micelle structures when dissolved in an aqueous electrolyte solution. Typically, the ionic and/or hydrophilic head groups of surfactants (*e.g.* sodium dodecyl sulfate, SDS) form the surface of the micelles, while the hydrophobic tails occupy the core. When an electric field is applied, the analytes are separated according to the differences in their partition between the bulk aqueous solvent and the micelles [12]. The separation process involves hydrophobic as well as other non-specific interactions [11]. The mass distribution ratio in MEKC, k_{MEKC}, is defined as [2]

$$k_{MEKC} = \frac{n_{mc}}{n_{aq}} = K\frac{V_{mc}}{V_{aq}} \tag{8}$$

where n_{mc} and n_{aq} are the molar amounts of the analyte in the micellar and aqueous phases, respectively, K is the distribution constant and V_{mc} and V_{aq} are the corresponding volumes of the phases.

Since no solid stationary phase is employed, Terabe [3] describes MEKC as a type of liquid–liquid partition chromatography. According to Khaledi [11], MEKC combines some of the features of reversed phase HPLC and CE. Because the dynamic aggregates possess an electrophoretic mobility in the capillary, the micellar solution is called a pseudostationary phase. Consequently, a limited elution window exists. In successful MEKC analyses, the analytes migrate between the neutral EOF marker and the frontline of the pseudostationary phase (Figure 1).

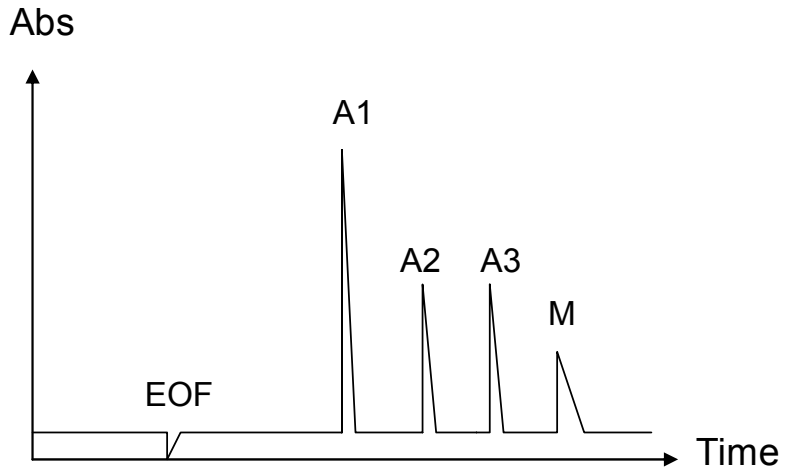

Figure 1. A schematic MEKC electropherogram (performed at normal polarity). EOF is the marker of the electroosmotic flow; A1, A2, and A3 are electrically neutral analytes; and M is the hydrophobic marker of the frontline of the micellar phase, consisting of anionic surfactants.

SDS is the most commonly used surfactant in MEKC [13]. Among its many appropriate features are high aqueous solubility, high aggregation number 62, relatively low CMC, and low UV absorptivity [11]. However, SDS micelles often fail to provide sufficient resolution between structurally similar compounds [14–16]. Selectivity in MEKC can be altered by modifying the composition of the pseudostationary phase. Mixed micelles, consisting of two or more types of surfactants, such as SDS and bile salts, often provide better resolution between structurally related steroid compounds [17].

Bile acids are compounds with a hydroxyl-substituted steroid backbone [14], which play an important role in lipid digestion and absorption, solubilization of cholesterol, and bile formation. According to Roda et al. [18], the number and orientation of hydroxyl groups in the bile acid structure determine its detergency (the higher the number of OH groups, the higher the CMC). Taurocholic acid, having three hydroxyl groups, possesses a CMC of 6 mM in 150 mM NaCl [18].

Unlike SDS, which forms large spherical micelles, bile salts form small, helical aggregates [14]. Bile salts are seldom employed as the only surfactants in MEKC, but rather to form mixed micelles.

Wiedmer et al. [15] have shown that sodium cholate (SC) in low concentrations incorporates into SDS micelles so that the hydrophilic (ionic) groups face the aqueous solution. They have also confirmed that the micelles change little in size with increase in the amount of SC. In other words, low concentrations of cholate molecules are dissolved in the SDS micelles as hydrophobic analytes. Further, Wiedmer et al. found that in a solution containing SDS (above its CMC) and SC, a wide range of micelles exist with different SDS-to-SC ratios [15]. The diversity of SDS-SC micelles is beneficial in the separation of corticosteroids and androstenedione by MEKC [15, 19]. Notably, with the SDS concentration kept constant (18 mM) while the concentration of SC is increased from 0 to 55 mM, the incorporation of corticosteroids and especially that of androstenedione in the SDS micelles is clearly diminished [15]. The authors explain this in terms of the competitive incorporation of SC and the steroids into the SDS micelles.

Excellent resolution for corticosteroids has also been achieved with mixed micelles consisting of SDS and other bile salts, *e.g.* SDS / sodium taurocholate [16], SDS / sodium dehydrocholate [16], and SDS / taurocholate / glycodeoxycholate [14]. The MEKC applications for testosterone and closely related compounds are few by comparison [20–23], and none of them relies on the use of mixed micelles. Exhaustive studies in this field are clearly desirable.

MEKC can be employed in sample concentration by the sweeping effect. Capillary electromigration techniques typically suffer from low sensitivity owing to minute sample volumes and a limited optical path length (*i.e.* i.d. of the capillary), which means that in-capillary concentration is highly beneficial. Sweeping results when the pseudostationary phase, while penetrating the sample zone,

entraps and accumulates the analytes [24, 25]. The sample is prepared in a solution that is free from the pseudostationary phase and has conductivity similar to or higher than that of the background electrolyte (BGE) [25]. Effective sweeping can be achieved when the analytes interact markedly with the pseudostationary phase [26].

Theoretically, following the law of conservation of mass, the analyte concentration (C_{sweep}) after sweeping is [24]

$$C_{sweep} = C_{inj}(1 + k) \tag{9}$$

where C_{inj} is the concentration of analyte in the injected sample zone and k is the retention factor.

Usually, the effectiveness of sweeping is expressed as a sensitivity enhancement factor in terms of peak height (SEF_{height}) [27].

A higher sensitivity enhancement is achieved under acidic conditions (pH 2–3) than under neutral and alkaline conditions [28] owing to the attenuated EOF. A schematic view of sweeping of hydrophobic neutral steroids under neutral or alkaline conditions is presented in Figure 2.

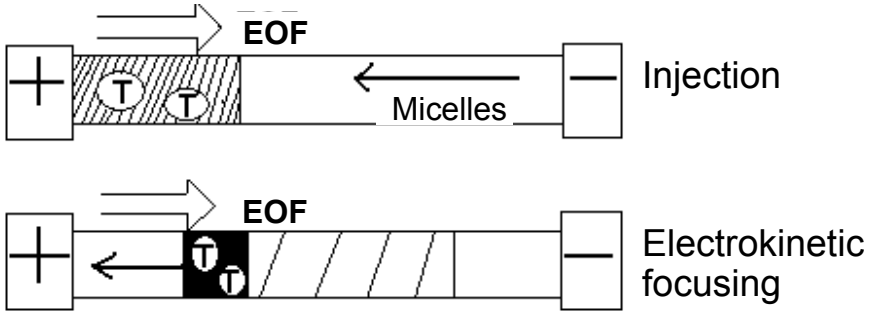

Figure 2. Sweeping of hydrophobic neutral steroids (T) with anionic surfactants (micelles) under neutral or alkaline conditions. A relatively large sample volume is injected from the anode side. The micelles, having absolute electrophoretic mobilities toward the anode, penetrate the sample zone, entrap and accumulate the analytes. Modified from Ref. 29.

Through sweeping between 100- and 5000-fold [24, 30, 31] concentration of hydrophobic analytes has been achieved under acidic conditions. At reversed polarity, a 1500-fold concentration of testosterone was achieved by sweeping with 100 mM SDS in 100 mM phosphoric acid (pH 1.9) containing 20% ACN and 2% MeOH [24]. In another approach, a 330-fold concentration of T was achieved with a pseudostationary phase consisting of 20 mM SDS / 60 mM *N*-dodecyl-*N,N*-dimethylammonium-3-propane-1-sulfonic acid solution in 10 mM phosphate under neutral conditions (pH 7.0) [32].

Hitherto, sweeping has mostly been investigated and utilized in UV spectrophotometric detection [24, 30–32] and LIF [33]. Rarely has it been applied with MS detection. Isoo et al. [28] demonstrated the use of sweeping in MEKC-APCI-MS analyses of cationic and uncharged compounds under acidic and neutral conditions. Under neutral conditions, about 100-fold increase in the concentration was achieved for aromatic amines, whereas under acidic conditions, 100 to 600-fold sensitivity enhancement was obtained for amines and alkyl phthalates [28].

Partial filling micellar electrokinetic chromatography (PF-MEKC) was derived from micellar electrokinetic chromatography (MEKC) by Terabe et al. [34, 35] as a means to improve the performance of in-line mass spectrometric (MS) detection after the separation of neutral analytes by MEKC. The surfactants employed in MEKC are poorly compatible with MS detection, for many reasons. Nonvolatile surfactants easily contaminate the MS instrument. They tend to make the electrospray unstable [36–38], and they often lower the sensitivity of the detection [35, 39–41]. In PF-MEKC, only part of the capillary is filled with the micellar pseudostationary phase solution. Anionic surfactants, which are preferred in PF-MEKC, have a lower total velocity to the detector than do the analytes. When an electric field is applied, the analytes separate while passing through the micellar solution. However, after passing the plug of the pseudostationary phase, the analytes migrate in the background electrolyte along with the EOF, *i.e.* faster than the surfactants. The electric voltages are switched off immediately after identification of the analytes, and the capillary containing the surfactants is then rinsed before the next analysis [37].

The surfactant concentration and the micellar zone length are the main experimental parameters that can be varied to optimize resolution in PF-MEKC

[42]. The behavior of the injected micellar solution differs from that in traditional MEKC. In general, micelles possess a much higher effective mobility than the individual surfactant monomers because micelle formation leads to reduced 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 [42]. Since the amount of micelles decreases with time, compounds with a higher retention factor will experience a smaller effective separation length. One notable drawback of PF-MEKC-MS is that efficiencies tend to be lower than with normal MEKC [42].

It has been suggested that comparable migration times can be obtained by varying the micellar zone length and surfactant concentration in different ways [42]. In PF-MEKC, however, an additional band broadening occurs at the micellar zone-buffer boundary due to the partial laminar flow, generated by the difference in the electric field strengths of the two zones [42]. Since this band broadening increases with higher retention factors, a low surfactant concentration is to be preferred.

Since the first studies of PF-MEKC-MS [35, 43], several applications of the technique have been presented, many of them relying on electrospray ionization (ESI). Identifications by PF-MEKC–ESI-MS have been reported for corticosteroids [37], peptides [35], herbicides [43], pesticides [44], pharmaceuticals [39, 45, 46], iridoid glucosides in plant samples [47], and microbial signaling molecules (*N*-acyl-L-homoserine lactones) in microbial extracts [48]. The applications for human biomolecules are still few [35, 37], as well as those for real samples in general [47, 48].

2.1.3 Electrokinetic chromatography and cyclodextrin-modified micellar electrokinetic chromatography

Electrokinetic chromatography (EKC) comprises capillary electromigration techniques in which additives are employed as components of a dispersed phase moving at a velocity different from the ν_0 [2]. According to IUPAC, MEKC is a

special case of EKC [2]. Other useful additives, besides surfactants, especially for the separation of enantiomers, are cyclodextrins (CDs), including underivatized and natural CDs such as α-, β-, and γ-CDs, and various derivatized CDs [27]. CDs are widely utilized as additives in MEKC [27, 49, 50, 51]. For instance, Otsuka et al. [27] describe the enantiomeric separation of triadimenol with 25 mM hydroxypropyl-γ-CD / 50 mM SDS in phosphate buffer (pH 7.0). They also investigated the sweeping effect under optimized conditions and found an approximately 10-fold increase in terms of the sensitivity enhancement factor measured as peak height (SEF_{height}). Recently, a brief report describing the separation and in-capillary concentration of corticosteroids by MEKC was published by Kartsova [52]. The sample was prepared in BGE (25 mM phosphate, pH 2.5) containing 5 mM of β-CD, and the pseudostationary phase consisted of 20 mM SDS and 4.5 mM urea. When β-CDs were added to the sample matrix, SEF_{height} values between 20 and 200 were obtained as compared to samples without CDs.

Only one study describes the separation of T and related structures by EKC and CD-modified MEKC. Urban et al. [22] investigated the determination of T, MT, and methandrostenolone with sodium taurodeoxycholate, neutral β-cyclodextrins (β-CDs), and/or anionic succinylated β-CDs as auxiliary components in 50 mM borate (pH 9.0). The best results were obtained with 10 mM succinylated β-CD and 1 mM β-CD in 50 mM borate not containing surfactants. Hitherto, no related reports exist.

2.1.4 Affinity and immunoaffinity capillary electrophoresis

According to IUPAC, affinity capillary electrophoresis (ACE) refers to those capillary electromigration techniques in which the BGE contains substances capable of specific interactions with the analytes [2]. Affinity interactions are often understood as selective binding between compounds at least one of which is a biopolymer (usually protein) [53]. The terminology for ACE has not yet been fully established. According to several authors [54–56], in ACE and specifically in immunoaffinity capillary electrophoresis (IACE, discussed below), the interacting molecules can be either free in solution or immobilized to a solid support.

From the analytical point of view, dynamic ACE and MEKC have a number of features in common, the most important being the noncovalent interactions occurring between the analytes and the electrolyte additives during separation. However, the separation mechanisms are radically different. In ACE, the affinity interactions, which are often highly selective [53], occur between *molecules*, whereas in MEKC, the analytes are usually retained by dynamic micelles through non-specific electrostatic and hydrophobic interactions.

Furthermore, except for a few competitive approaches [57–59], ACE has only been performed with charged analytes, ranging from proteins to hydrophilic carbohydrates [60, 61] and a variety of drugs. MEKC, in contrast, is usually applied for electrically neutral analytes of moderate hydrophobicity, although separation of charged analytes is also possible. ACE is typically performed in mild electrolyte solutions such as ones that mimic body fluids, whereas MEKC, performed with high concentrations of surfactant(s), is a destructive method for highly organized three-dimensional structures. Finally, ACE is usually utilized in determination of binding between xenobiotics and biomolecules, though separations of drug enantiomers with the aid of auxiliary proteins have also been reported [62–66]. In comparison with MEKC, ACE offers separations of much reduced resolution.

ACE is a convenient technique for evaluating association constants between drugs and proteins [58, 67–78]. In dynamic ACE, the equilibrium between free and protein-bound ligand (*e.g.* drug) is established in the capillary on the basis of electrostatic and hydrophobic interactions, reinforced by conformational compatibility. The kinetics of the interaction between a protein and ligand determines whether dynamic ACE can be used for evaluating association constants. If the interactions are very strong (irreversible in the CE separation time scale), nonequilibrium ACE can be employed, in which the sample containing both the protein and the ligand is pre-equilibrated, and the free and protein-bound ligand are then separated and quantified [79].

In the conventional dynamic approach, the ligand (*e.g.* a drug) is used as electrolyte additive and the protein as sample. However, this approach often has disadvantages as compared with the opposite approach, in which the capillary is filled with an electrolyte solution containing the protein in different concentrations, and a constant molar amount of ligand (drug) is introduced to the capillary. In

the latter case, after application of an electric field, the apparent mobility of the drug depends on the association (*i.e*. binding) constant, K_b, and the difference between the electrophoretic mobilities of the free and protein-bound drug. Variation in the concentration of the protein solution introduced to the capillary makes it possible to express the apparent mobility of the drug as a function of protein concentration, and to obtain a Scatchard plot with the assumption of an association stoichiometry of 1:1.

The benefits of using the protein as the electrolyte additive and the ligand as the sample include smaller errors in the analyte migration time due to generally better peak shape, higher reproducibility of the ligand migration time, and a larger shift in the mobility of the analyte [79] when the concentration of the protein solution is altered.

Unfortunately, when the capillary is completely filled with a protein solution, the sensitivity of UV detection is lost due to high background absorption. To prevent this problem, Valtceva et al. [80] introduced the partial filling technique, in which only a small plug of protein solution is introduced to the capillary. Tanaka and Terabe [62] were the first to apply the partial filling technique in ACE (PF-ACE). To date, several PF-ACE separations of chiral drugs [62–66], including oxprenolol [66], bupivacaine [64], and propranolol [65], have been reported. PF-ACE has also been used in the determination of the association constants between α1-acid glycoprotein and propranolol, mepivacaine, and chlorpheniramine [76] and between carbonic anhydrase B and arylsulfonamides [59, 71].

ACE has many benefits over other methods used for the determination of association constants. In contrast to surface plasmon resonance (SPR) and HPLC, in ACE the proteins are present in mobile form and the protein immobilization and regeneration procedures are avoided. Sample consumption is low, concentrations of the interacting compounds in the capillary are easily controlled [54, 68, 74], and the reliability of the experimentally determined association constants is good [68]. Finally, the interactions between drugs and proteins occur without the sterical hindrances that may result from protein immobilization. In other words, the values obtained by ACE indeed reflect the equilibrium as it exists in solution [81]. Notably, however, problems may arise when ACE is applied to proteins that have a high molecular mass (> 100 kDa) or high p*I* (> 8.4) owing to electrostatic adsorption on the silica capillary [58, 82].

Hitherto, if competitive approaches are excluded, a notable limitation of ACE has been the ability to study directly only the affinity between ionized compounds.

Immunoaffinity capillary electrophoresis (IACE) refers to those ACE approaches that take advantage of interactions between antibody proteins and antigens. In IACE, the separation power of CE is combined with highly specific affinity interactions [56, 81, 83–85]. The combination of immunochemistry and capillary electromigration techniques has attracted considerable attention, and today numerous IACE methods are available [55, 81, 85, 86]. IACE can be carried out in three formats [55], referred to below as i–iii (Table 1).

We recently reviewed the IACE procedures since 1998 for the analysis of body fluids and tissues for drugs, hormones and disease markers [86] and concluded that, to date, IACE format ii (Table 1) has been most widely employed. IACE format i was investigated in the present study, and special features of this format are discussed in the following. Readers with an interest in IACE in general are referred to Refs. [56], [81], and [86].

Table 1. Description of IACE formats.

i	Immobilizing antibody or antigen onto the inner wall of the capillary or onto a support material integrated in the separation path, and performing complexation during or before CE separation.
ii	Mixing of antibody and antigen in a vessel and allowing complexation to proceed to equilibrium before introducing the mixture to the CE capillary.
iii	Complexing free antigen with free antibody in the CE capillary. When the electrolyte solution contains the antibody, the sample contains the antigen, and *vice versa*.

A schematic representation of IACE format i is shown in Figure 3.

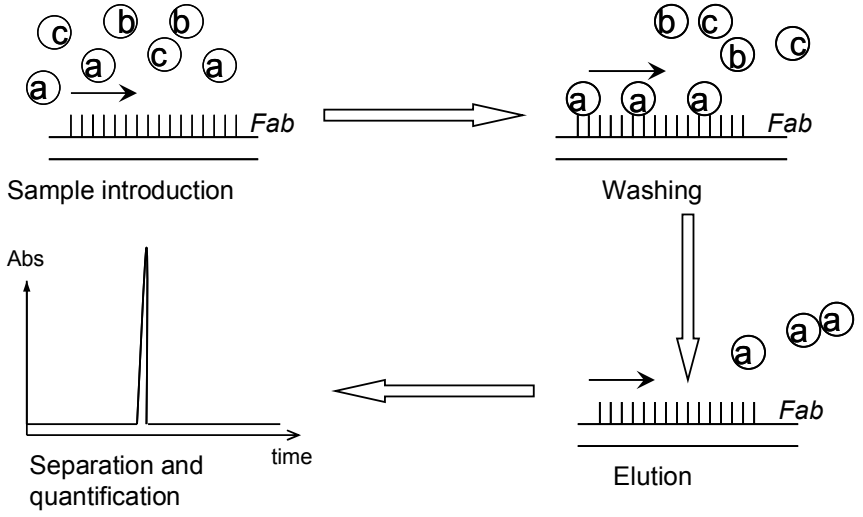

Figure 3. Schematic representation of IACE (format i). The capillary inner wall is coated with antibodies or antibody fragments, such as Fabs. The IACE procedure comprises sample introduction, washing, elution of the analytes (a), separation and quantification. Modified from Ref. 83.

Whenever IACE format i is employed, the orientation of the immobilized antibodies becomes of great concern [56]. Optimal antigen binding is achieved when the binding site of the antibody is oriented away from the capillary wall or other support material. Antibodies and immobilization procedures are further discussed in section 2.6.

Few IACE analyses have been performed with immobilized antibodies (i) [83, 84, 87–92]. Pioneering work in the field was done by Guzman and Phillips. The first IACE studies describing the determination of urinary methamphetamine and urate [91] and serum IgE [92] with use of immobilized antibodies were performed by Guzman et al.

In 1998 [83], Phillips presented an IACE method for the determination of some biologically important neuropeptides in human biopsy tissues. One-third of the silica capillary was coated with anti-neuropeptide antibody Fab' fragments. After pretreatment of the tissues (including labeling of the neuropeptides), a

sample of 100 μL was injected into the immunoaffinity capillary and incubated for 5 min. The capillary was then rinsed and the analytes were eluted at low pH. The analytes were identified by LIF. Excellent recoveries (over 95%) were obtained. Shortly thereafter [87], a similar approach was introduced for the determination of recombinant cytokines in body fluids, such as plasma, serum (equivalent to blood plasma except that it lacks the plasma components that have taken part in the clotting process, mainly fibrinogen), urine, saliva and cerebrospinal fluid.

Gonadotropin-releasing hormone (GnRH) in serum and urine specimens from a healthy person and from a patient suffering from benign prostatic hyperplasia was determined by Guzman [84], who used IACE coupled to a UV spectrophotometer and ESI-MS. GnRH present in the serum or urine was captured by immobilized monovalent Fab' fragments covalently bound to controlled porous glass (CPG) beads in a microscale-format affinity chamber. The capillary containing the GnRH concentrator was prepared to fit into a commercial CE cartridge. The limit of quantification (LOQ) for GnRH in serum was 1 ng/mL with the IACE-UV technique. GnRH was also identified by CE–ESI-MS.

A few years ago, Guzman [89] introduced a compact solid-phase microextraction device for use in IACE determinations of ibuprofen, naproxen, and the neuropeptides angiotensin II and neurotensin in spiked urine. Monomeric Fab' fragments against the analytes were covalently immobilized on CPG beads *via* hinge-region cysteins that allowed optimal orientation. The beads were placed at a crossing and fixed with porous polymeric frits. In the horizontal position there was a tube for sample introduction and buffer washing (performed manually with a syringe), while in the vertical position, two fused-silica capillaries were connected to the crossing to perform CE. Diluted urine specimens were spiked with the drugs or neuropeptides (1–5 ng/mL), and the samples were analyzed in two separate microconcentrators. Identification of the separated analytes was performed by UV. The LOQs after spiking of the matrices were approximately 1 ng/mL for ibuprofen and naproxen, and 0.5 ng/mL for the neuropeptides.

Clinically relevant levels of the disease marker cardiac troponin I (cTnI) in serum were determined by Dalluge et al. [88], who used a short bed of monoclonal anti-cTnI-coated porous silica in a fused-silica capillary. The porous silica was first chemically modified with 3-aminopropyltriethoxysilane, dried,

and exposed to glutaric dialdehyde. Subsequently, anti-cTnI antibodies were randomly immobilized to glutaric dialdehyde *via* free amino groups. The immunoaffinity matrix was introduced into the capillary by pressure and held in the capillary by two frits. Detection was performed by UV. The method proved to be sensitive (LOD < 10 ng) and precise (RSD 3–4%). According to the group, the only limitation of the technique was the low flow rate through the capillary resulting in long sample injection times (~ 60 min).

A common feature of the above-reviewed IACE (format i) applications is that they were developed for charged analytes. As of today, IACE applications (format i) for neutral analytes do not exist.

In recent years, the focus in IACE has turned to micro- total analysis systems [93–99]. In these applications, the whole procedure, including immunoaffinity extraction, optional labeling reactions, electrodriven separation, and identification, is performed on a microchip. Microchip technology and the phenomena affecting the movement of solutions and analytes in microfluidic devices differ from those of capillary electromigration techniques, but preliminary macroscale IACE studies may offer a valuable background for the microscale applications.

2.2 Testosterone and related steroids

The capillary elecromigration methods developed in this work were applied to the analysis of testosterone and related steroids.

Andrew [100] classifies steroid hormones by their biological activity into androgens, estrogens, progestogens, corticosteroids, and mineralocorticoids. Our focus was on androgens, a group consisting of testosterone and related hormones and their synthetic derivatives.

Testosterone (T) is the principal androgen, produced in men mainly by the testis and in women by the ovary and the adrenal gland [101]. Testosterone has both anabolic and androgenic effects in the human body. Anabolic effects stimulate nitrogen fixation and erythropoiesis, and increase protein synthesis (Figure 4) [4, 102]. Testosterone is involved in prenatal androgenic sexual differentiation,

sexual maturation, and fertility throughout male life [102, 103]. It acts on a number of target tissues, including bone, skeletal and cardiac muscles, brain, liver, kidney, and adipocytes [4]. In recent investigations of the role of T in the development of metabolic syndrome and diabetes in men, it is proposed that low testosterone levels promote these metabolic disorders [104, 105].

Natural testosterone concentrations in plasma range from 8–35 nM in healthy adult males down to less than 2 nM in women and children [103, 106]. Only about 2% of T is free in plasma [107, 108]. Most T is conjugated with human serum albumin (HSA) (ranging from 50 [107] to 54% [108] in men, and from 20 [107] to 30% [108] in women) and with sex hormone binding globulin (SHBG) (44% and 78% in men and women, respectively, as tested in Canadians) [108]. Free and albumin-bound T are considered to be bioavailable [108, 109].

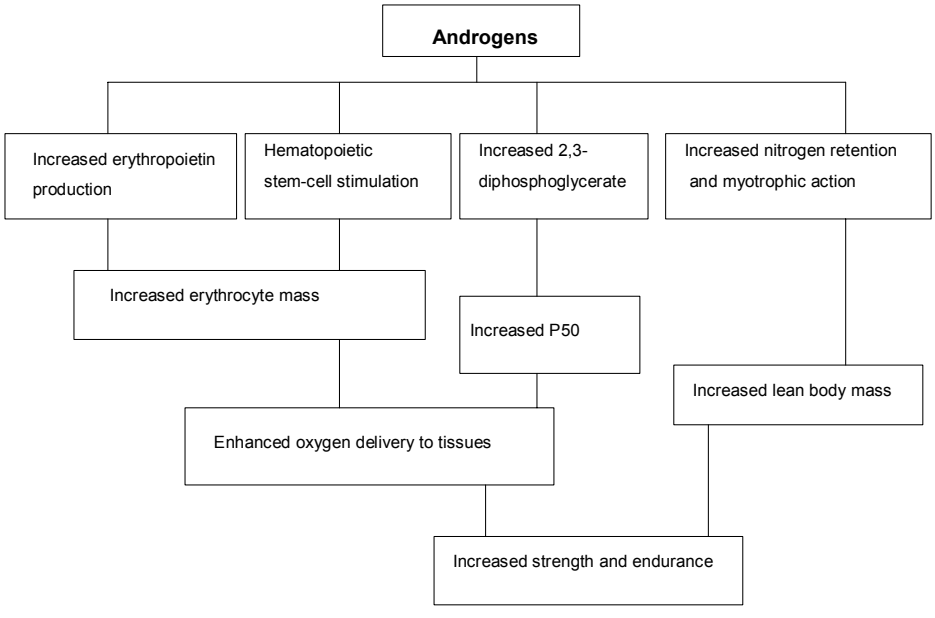

Figure 4. Combined effect of androgens on the oxygen delivery system and muscle mass (myotrophic action). 2,3-Diphosphoglycerate decreases hemoglobin–oxygen affinity. P50 = oxygen pressure at which 50% of hemoglobin is oxygen saturated under normal physiologic conditions. Modified from Ref 4.

Binding of testosterone to HSA has been widely investigated [107, 110, 111–114]. It is suggested that there is one primary binding site for T in the II domain of the

HSA structure [112, 113] and that the stoichiometric binding ratio is 1:1 [113]. Association constants (K_b) for T and HSA of (2.0 ± 0.2) $\times 10^4$ M^{-1} (at pH 7.4, 25 °C) [113], (2.8 ± 0.2) $\times 10^4$ M^{-1} (at pH 7.4, 20 °C) [112] and (3.3 ± 0.3) $\times 10^4$ M^{-1} (at pH 7.4, 25 °C) [114] have been reported. Binding of T to HSA is affected by temperature, pH, and the presence of other plasma components, such as fatty acids [113, 114]. Interestingly, the affinity between T and bovine serum albumin (BSA) is slightly weaker than that between T and HSA [114].

Testosterone (Figure 5a) and related steroids have a hydrophobic tetracyclic perhydro-1,2-cyclopentanophenanthrene ring in their structure. T and many related steroids are largely metabolized in the human body before excretion in urine [101]. Enzymatically catalyzed phase-I reactions convert the steroids into more polar structures in order to inactivate them and facilitate their elimination from the body [101]. Phase II reactions act to couple the steroid or its metabolite with glucuronic acid or sulfate [101]. 3α-*O*-β-Glucuronides are the predominant metabolites of T and structurally similar compounds. A tiny fraction of testosterone is also excreted as sulfate conjugate [115, 116]. Normally, intact T is not found in urine [117]. After a successful deconjugation of glucuronide groups, the concentration of T in male urine is approximately 30–60 µg/L [118, 119].

Androstenedione (A) (Figure 5b) is an endogenous immediate precursor of testosterone. In healthy men and fertile women, the clinical reference values in plasma vary from 3 to 7 and from 3 to 11 nM, respectively. The metabolism of orally administered androstenedione and urinary excretion of androstenedione and testosterone metabolites have been investigated [120]. It was concluded that the administration of androstenedione increases the excretion rate of conjugated testosterone, and that androstenedione is largely metabolized before release into general circulation.

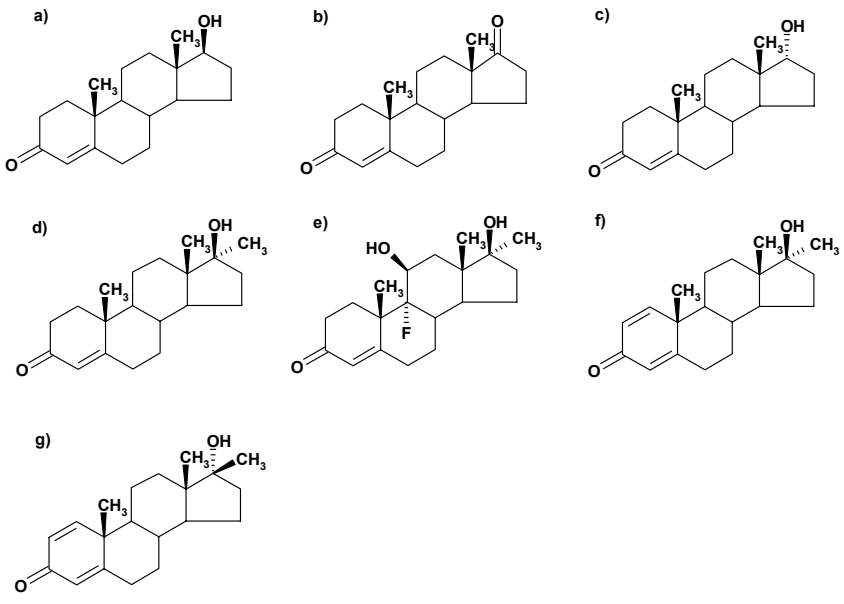

Figure 5. Structures of investigated steroids: a) testosterone, b) androstenedione, c) epitestosterone, d) methyltestosterone, e) fluoxymesterone, f) metandienone, and g) (17)-epimetandienone.

Epitestosterone (E) (Figure 5c) is an endogenous epimer of testosterone. It is excreted from the body mainly as glucuronide conjugate [116]. For a long time it was assumed to be a metabolite with no hormonal activity [121]. In recent years, however, the physiological role of epitestosterone has been more thoroughly revealed, and it is considered to have antiandrogenic activity [121, 122]. Concentrations of epitestosterone in plasma average 2.5 nM in men and 1.2 nM in women [121]. After deconjugation of glucuronide groups, the concentration of free E in urine is approximately 30 µg/L [118, 123].

The desirable anabolic effects of testosterone have encouraged the synthesis of over 100 testosterone-related compounds for use as drugs and doping agents [4]. Together these compounds are called anabolic androgenic steroids (AASs). Methyltestosterone (Figure 5d), fluoxymesterone (Figure 5e), and metandienone (Figure 5f) are commonly used AASs. AASs are employed as therapeutic agents in the treatment of chronic diseases such as hypogonadism, endometriosis, cancers, liver and renal failures, and HIV [102]. The use of testosterone, epitestosterone, and AASs as doping agents is prohibited by the Medical Commission of the

International Olympic Committee, but their use among athletes is frequently revealed [124]. The concentrations of T, E, and AASs in urine samples are determined in doping control. E is of great interest as the denominator of T/E, a marker of T administration [125]. The current cut-off value for T/E, set by the World Anti-Doping Agency, is 4 [124].

AASs often undergo a more concise metabolizing pathway than T [101, 126]. Methylation at position C-17α, for instance, blocks the metabolism of the D-ring (comprising 5 carbon atoms) [101]. Glucuronides are the major metabolites of most AASs [101]. The few exceptions include fluoxymesterone and metandienone [101]. After oral administration, metandienone is excreted as two unconjugated metabolites, namely 17-epimetandienone (Figure 5g) and 6β-hydroxy-17-epimetandienone [101].

2.3 Conventional techniques for the analysis of testosterone and related steroids

The conventional techniques for the determination of testosterone and related steroids include immunoassays, GC–MS, LC, and LC–MS. These are briefly described below.

2.3.1 Immunoassays

Many clinical laboratories use commercial immunoassay kits to determine the concentration of bioavailable (non-SHBG-bound) and total testosterone in plasma or serum [108, 127]. Immunoassays are rapid high-throughput methods that take advantage of specific interactions between antibody proteins and antigens. An antigen must be efficiently bound by the antibody, *i.e.* the antibody must possess high affinity to the antigen. Immunoassays can be divided into those that employ labeled reagents and those that employ non-labeled reagents. Labels are typically radioactive or chemiluminescent molecules [106]. T-specific immunoassays are usually performed by using a competitive approach [106, 127]. The sample containing T is mixed with a solution containing a known amount of labeled T and an anti-testosterone antibody. During incubation, labeled T and T originating in the sample competitively bind to the antibody [106]. Testosterone concentration is inversely related to the amount of signal caused by the bound labeled T.

The analytical sensitivity of most total testosterone assays is about 0.5 nM (0.14 μg/L) [108], which allows reliable determination of T in healthy adult males [106, 108]. This sensitivity does not allow quantitative determination of T in women or prepubertal boys, especially if they have abnormally low levels of T [108, 127]. Another common problem of immunoassays [128], and especially of steroid assays [106, 126, 129, 130], is insufficient specificity of polyclonal antibodies. Polyclonal antibodies, on which most commercial immunoassays rely [131], often cross-react with structurally similar compounds, such as epitestosterone and dehydroepiandrosterone (DHEA), leading to false (too high) results in T determinations [106].

2.3.2 GC–MS

Gas chromatography- mass spectrometry (GC–MS) is another established analytical method for testosterone and related compounds, in both clinical and doping control applications [106, 120, 125, 126, 132–138]. GC–MS is a highly sensitive method, providing limits of detection at the μg/L [136] and even ng/L [115] level for derivatized steroids. High accuracy can be obtained by using isotope-labeled steroids as internal standards [126]. Sensitivity and selectivity can be improved by using GC–MS/MS instrumentation. However, GC–MS analyses of steroids require laborious sample pretreatment, including extraction (SPE or LLE), deconjugation of phase II metabolites, and derivatization of the polar hydroxyl and carbonyl groups [126, 134, 138]. Non-specific LLE and SPE performed, for example, with neutral polystyrene resin or stronger reversed phase bound silica particles [126] are not suitable for selective extraction of steroid molecules. Moreover, the mandatory derivatization procedures may be incomplete, leading to multiple derivatives of polyfunctional compounds [134].

2.3.3 LC and LC–MS

Liquid chromatography [123, 139–142], and especially liquid chromatography–mass spectrometry (LC–MS) [116, 117, 119, 135, 143–149] are of growing interest for steroid analysis. LC and LC–MS are more straightforward methods than GC–MS since derivatization is not required [142–144, 146, 149]. As in GC–MS, improvement in selectivity and sensitivity is achieved with MS/MS

instrumentation [116, 118, 144–148]. For example, a LOD of 1.5 ng/L is reported for T [147] and a LOQ of 0.5 µg/L (1.7 nM) for serum T [144]. A strong disadvantage of all the HPLC applications is the compulsory use of organic solvents, which tend to be both toxic and expensive.

2.4 Analysis of testosterone and related steroids by capillary electromigration techniques

The application of capillary electromigration techniques to the separation of testosterone and related compounds is a relatively new approach. Reports describing the separation of at least two of the compounds involved in this work are reviewed below. The most relevant data of these studies is compiled in Table 2. Note: only a few of the applications have been tested for quantitativity.

In 1996, Fernandez et al. [20] reported a MEKC separation of liver microsomal testosterone products. The method was based on a complex separation medium consisting of 85% of 47.5 mM Tris-HCl / 2.5 mM borate / 60 mM SDS / 15 mM β-cyclodextrin and 15% of EtOH (v/v). Androstenedione, testosterone, and seven testosterone metabolites were separated in 18 min. The LOD for T was 0.4 µM (115 µg/L). However, repeatability was relatively poor, the absolute inter-day RSD values for migration times and peak areas being close to 10%.

In 1999, Kuei et al. [21] presented a brief report on the effect of three organic modifiers (ACN, MeOH, and 1-propanol) on the MEKC separation of seven steroids, including testosterone and methyltestosterone. They tested mixtures of 10% (v/v) of an organic modifier in borate-phosphate electrolyte solutions containing 20 mM SDS. The use of ACN and 1-propanol in 60 mM borate-30 mM phosphate resulted in baseline separation of all seven steroids in 17 min at best. The concentrations of borate and phosphate were observed to have a strong influence on resolution, with high concentrations (80 mM borate / 40 mM phosphate) leading to better resolution. The enhanced resolution at high concentration was explained by decrease of the EOF. Validation of the method was not presented.

In 2005, Urban et al. [22] introduced a separation and in-capillary preconcentration method for three anabolic steroids, including T and MT. In place of SDS, they

used sodium taurodeoxycholate, neutral β-cyclodextrins (β-CDs), and/or anionic succinylated β-CDs as auxiliary components in 50 mM borate (pH 9.0). Best results were obtained with 10 mM succinylated β-CD and 1 mM β-CD in 50 mM borate devoid of surfactants. In other words, electrokinetic chromatography (EKC) was preferred over MEKC. The LODs for T and MT after a reverse migrating sample stacking process were 100 and 84 μg/L, respectively. The method showed good linearity for all steroid standards (r^2 = 0.990 or higher).

Recently, Silva et al. [23] investigated the separation of 11 steroids, including T and MT, by MEKC using EtOH, ACN, and/or tetrahydrofuran as additives in 20 mM SDS / 20 mM tetraborate (pH 9.4). A favorable separation was obtained with an addition of 20% (v/v) EtOH to the pseudostationary phase. Validation of the method was not presented.

A few capillary electrochromatographic (CEC) applications for testosterone and related steroids have been published [150, 151]. Huber et al. [150] demonstrated the separation of T, A, and four other steroids in 6 min by applying gradient elution in CEC. The silica capillary (i.d. 50 μm, L_{tot}/L_{packed} 17.6/9.6 cm) was packed with Zorbax ODS particles (mean i.d. 3.5 μm), and a voltage of 14 kV was applied across the capillary. Another CEC approach was reported by Seifar et al. [151], who used silica capillaries (i.d. 50 μm, L_{tot}/L_{packed} 32/24 cm) packed with even smaller porous Zorbax ODS particles (mean i.d. 1.8 μm). After the application of a voltage of 25 kV, separation of T, MT, and six other steroids was completed in 5 min. Unfortunately, validations of the CEC methods were not presented [150, 151].

During the past few years, the group of Riekkola and Wiedmer has been investigating the use of mobile liposomes in capillary electromigration techniques [152, 153], and liposome coated silica capillaries in CEC [154–159]. T, A, and a few other steroids have been used as model compounds. The separations of steroids with mobile liposomes were efficient and repeatable (RSD of migration times 1.8%, n = 6) [152]. Separation of the same steroids in CEC studies was further investigated, and in one of the reports, T and A were separated in just 4 min [158]. However, linearity and sensitivity of these methods remain to be investigated to evaluate their potential in quantitative analyses.

Table 2. Capillary electromigration applications for testosterone and related structures.

Analytes	CE method	Special features (if any)	Det. (nm)	LOD	Linearity	Ref.
A, T	MEKC		240	0.4 µM (115 µg/L) for T	n. r.	[20]
T, MT	MEKC			n. r.	n. r.	[21]
T, MT	MEKC, EKC		245	100 µg/L for T, 84 µg/L for MT	$r^2 = 0.990$	[22]
T, MT	MEKC		247	n. r.	$r^2 = 0.992$ for T, $r^2 = 0.991$ for MT	[23]
A, T	CEC		205	n. r.	n.r.	[150]
T, MT	CEC		254	n. r.	n.r.	[151]
A, T	EKC	Use of mobile liposomes	245	n. r.	n. r.	[152], [153]
A, T	CEC	Use of immobilized liposomes	245	n. r.	n.r.	[154], [155], [156], [157]

n. r. = not reported

2.5 Conventional sample pretreatment in the analysis of body fluids for testosterone and related compounds

The presence of steroids at low concentration in complex body fluids, such as urine, plasma, and serum, makes sample pretreatment compulsory in all steroid determination procedures, excepting certain immunoassays [127]. In many immunoassays, release of steroids bound by SHBG is necessary [106]. Analytical techniques such as GC–MS, LC, LC–MS, and capillary electromigration techniques require sample pretreatment before separation, including deconjugation of the phase II conjugates, one or more extractions, and even derivatization (GC–MS). The following sections describe typical hydrolysis reactions and a few representative non-specific extractions to give the reader an overview of these procedures. Derivatization is not discussed since it was not applied in the present study.

2.5.1 Deconjugation of phase II metabolites

Deconjugation of the phase II metabolites of T and related structures is often performed as part of the sample pretreatment. The most common approach is enzymatic hydrolysis. The structures of the glucuronide and sulfate conjugates of testosterone are shown in Figure 6.

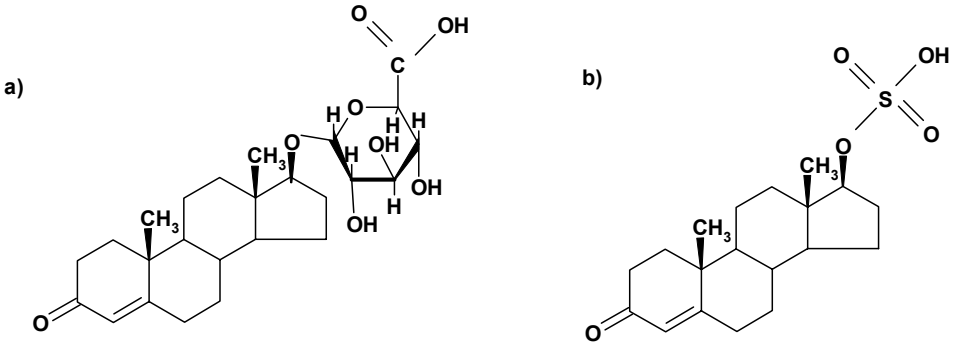

Figure 6. Structures of the glucuronide and sulfate conjugates of testosterone. Modified from Ref. 101.

The juice of *Helix pomatia (Hp)* contains both *β*-glucuronidase and arylsulfatase activities, and has been applied, for example, in the determination of a wide range of anabolic steroids in bovine urine [160] and meat [161] by GC–MS. Typically, the hydrolysis reaction with *Hp* is performed for several hours under slightly acidic conditions (pH 5.2–5.5) at 52–55 °C [160, 161]. As a serious disadvantage, the juice of *Hp* may cause steroid conversion, for instance DHEA conversion to androstenedione [162] and androstenediol conversion to T [116].

Many sulfate conjugates are resistant to enzymatic hydrolysis, especially those possessing conjugates at C17 position [163]. As an alternative, they can be deconjugated with HCl under highly acidic conditions, but often their presence is simply overlooked [116].

The most common approach to deconjugation at present is to hydrolyze only the predominant glucuronide conjugates of T and related structures using *β*-glucuronidase, which usually is extracted from *E. coli* (Table 3). The amounts of T and E released from the glucuronide conjugates give the T/E ratio, which is

currently used in doping control to determine whether or not an athlete has abused steroids [124]. In comparison with hydrolysis by *Hp* juice, time is often saved, and interferences present in the complex *Hp* juice as well as undesired conversion reactions are avoided.

Table 3. Enzymatic hydrolyses of testosterone and related steroids by E. coli.

Matrix	Glucuronide conjugates	pH	Enzyme origin, units/ mL urine	Temp. (°C)	Incubation time	Ref.
male urine	T and E	6.8	*E. coli* ≥ 50	37	overnight	[132]
male urine	T and E	7	*E. coli* unknown	55	1 h	[118], [164]
human urine	T and E	7.0	*E. coli* ≥ 3.3	60	1 h	[135]
male urine	T and E	7.0	*E. coli* unknown	60	1 h	[165], [166]
male urine	E	7.0	*E. coli* > 4	60	1 h	[167]
human urine	T and E	6.9	*E. coli* unknown	55	1 h	[123], [149]
human urine	T	5.5	*Helix pomatia* unknown	37	overnight	[130]

2.5.2 Solid-phase extraction

Solid-phase extraction (SPE) is widely applied in the determination of steroids in body fluids [117, 118, 164–166, 168]. Though a simple and efficient method, SPE suffers from poor selectivity [169] and the compulsory use of organic solvents (*e.g.* methanol [117, 118, 148], diethyl ether [164, 167], hexane / diethyl ether [161], or dichloromethane / ethyl acetate (4/1, v/v [146]).

Aguilera et al. [167] applied solid-phase extraction (SPE) before enzymatic hydrolysis of epitestosterone glucuronides. Bond-Elut C_{18} cartridges were used, and after deconjugation the steroids were further extracted with diethyl ether by LLE. After evaporation of the solvent, the steroids were derivatized and analyzed by GC–MS.

Recently, Rauh et al. [148] presented an in-line extraction method for A, T, and 17α-hydroxy-progesterone combined with LC–MS-MS. 100 µL of sample (plasma, serum, or saliva) was mixed with an internal standard solution, equilibrated, and exposed to protein precipitation with MeOH / zinc sulfate (50 g/L) (1/1, v/v) solution. The clear supernatant was injected to the HPLC system equipped with an extraction column (Oasis HLB®, a copolymer consisting of lipophilic divinylbenzene and hydrophilic *N*-vinylpyrrolidone). The extraction column was washed with an aqueous solution and then, the bound material including steroids was eluted from the extraction material with MeOH / ammonium acetate (70/30, v/v) and directed onto the analytical column.

2.5.3 Liquid–liquid extraction

Liquid–liquid extraction (LLE) is a laborious procedure that is difficult to automate [170]. Moreover, it often suffers from reduced recovery as compared with SPE [164]. As well, the use of organic solvents such as hazardous methylene chloride [171], dichloromethane [20, 164], or diethyl ether [118, 136, 167] is typically required.

Marcos et al. [139] described the determination of testosterone and epitestosterone in human urine by capillary LC. A sample volume of 1.0 mL was required. After enzymatic deconjugation of the phase II steroid metabolites, $NaHCO_3$-Na_2CO_3 was added to the mixture and the steroids were extracted with *n*-pentane. The organic fraction was washed with KOH and evaporated to dryness. The residue was then reconstituted in a mixture of H_2O/ MeOH (80/20, v/v) and analyzed by LC.

Recently, another approach to extract T and E in human urine by LLE was described by Liu et al. [149]. A urine sample of 5.0 mL was required per analysis. After deconjugation of the glucuronide derivatives by enzymatic hydrolysis, the proteins were precipitated with trichloroacetic acid, and the supernatant was mixed with 2-propanol and K_3PO_4. The mixture was vortexed and centrifuged to facilitate phase separation, and the upper phase was analyzed by HPLC–MS. The extraction efficiencies for T and E were 92–102%. An advantage of Liu's method is the use of 2-propanol, which is less hazardous to the environment than *n*-pentane, for instance.

2.6 Steroid-specific solid-phase extraction

Conventional non-specific steroid extraction methods, discussed above, suffer from poor selectivity. An interesting alternative for non-specific SPE is immunoaffinity solid-phase extraction (IA-SPE). IA-SPE refers to those SPE procedures that rely on the high affinity and high selectivity of immobilized antibody proteins to target antigens present in a sample matrix [169]. IA-SPE enables selective or even specific extraction, concentration, and purification of analytes in complex matrices in a single step. Modern antibody engineering and production techniques are reviewed below, with special emphasis on studies involving anti-testosterone antibodies and Fab fragments. The theory of IA-SPE and applications for steroids are briefly presented.

2.6.1 Antibody engineering and production techniques

Antibodies (also called immunoglobulins) are binding proteins produced *in vivo* by B-lymphocytes. Their function is to provide specific affinity for foreign macromolecular substances, *i.e.* antigens. An intact antigen that is capable of activating the immune system is called an immunogen.

Antibody-antigen complexes are typically stabilized by a variety of dipole-dipole, van der Waals, hydrogen bond, and hydrophobic interactions. Owing to their specific and high affinity for antigens, antbodies have been widely used in analytical applications. Antibodies are able to capture antigens from highly complex matrices, such as body fluids, and thus they are especially useful in sample pretreatment procedures where eliminating interference from the sample matrix is desired.

Antibodies for analytical purposes have conventionally been produced in animals by immunizing them with antigens. Small compounds (haptens), such as many drugs, are usually unable to elicit antibody production unless attached to a macromolecular carrier, such as BSA. Antibodies produced in an animal are polyclonal, *i.e.* structurally heterogenic, and possess different affinities and specificities for antigens [172]. Often they cross-react with structurally similar compounds, which, unless carefully compensated, may cause serious problems in analytical applications such as IACE [173–175] and immunoassays [106, 126, 128–130].

Polyclonal antibodies, which contain two identical antigen binding sites, can be cleaved into fragments that are more suitable for analytical use. For instance, enzymatic digestion with papain results in two Fab fragments and one Fc fragment (Figure 7).

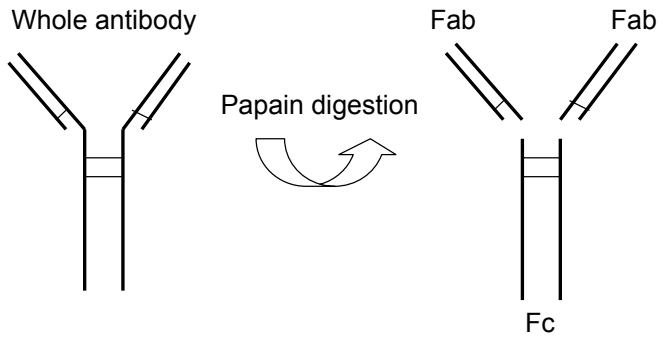

Figure 7. Schematic view of preparation of antibody Fab and Fc fragments by enzymatic digestion. Modified from Ref. 85.

Each Fab fragment contains one antigen binding site. Fab fragments are often preferred over whole polyclonal antibodies in analytical applications because differences in the p*I* value of polyclonal antibodies due to the variation in the number of sialic acids attached to the Fc region are then avoided [173]. Fab fragments deriving from polyclonal antibodies do not, however, solve the problem of cross-reactivity.

Some 30 years ago, Köhler and Milstein [176] introduced the hybridoma technique, which enables the production of single monoclonal (*i.e*. structurally identical) antibodies in cell cultures. The hybridoma technique usually commences with the immunization of mice with one (or more) desired antigen(s). Subsequently, immortalization of the immune response is performed by hybridization of isolated mice lymphocytes with myeloma (cancer) cells. Typically, a few hundred hybridoma cells are cloned, some of which are likely to produce the desired types of antibodies [177].

In 1988, Parmley and Smith [178] introduced the phage display technique, which provides the display of antibody (fragments) on the surface of a bacteriophage (virus that infects bacteria, e.g. *E. Coli*) as a part of phage's coat protein. Phage display allows one or a broad panel of species to be immunized,

and the genetic material encoding the antigen binding region of antibody (fragments) can be genetically engineered. Combining phage libraries prepared from various immunized animals leads to very large antibody libraries (up to 10^9 clones), from which desired antibodies can be enriched by specific panning strategies [177, 179]. An example of using phase display to produce recombinant Fab fragments is presented by Pulli et al. [179]. An attractive feature of the phage display technique is that it diminishes the need for ethically problematic animal immunization [179]. The use of the phage display technique in clinical method development has been described by several groups [179–181].

In vitro antibody engineering provides excellent tools for tailoring antibodies or their fragments in respect of affinity, specificity, and performance for specific applications [182]. *In vitro* production of antibody (fragment) against testosterone, however, is highly challenging. The binding of T to anti-T antibodies is almost exclusively due to shape complementarity because T is capable of participating in only a few hydrogen bonds [183]. Anti-T antibodies often cross-react with structurally similar compounds such as androstenedione, 5α-dihydrotestosterone, dehydroepiandrostenedione sulfate [183], and epitestosterone [184].

For immunoassays, the specificity of anti-T antibodies is crucial since the number of closely related steroid hormones in serum (and urine) is high, and their concentrations, usually very low, can vary significantly even among healthy individuals. Monoclonal antibodies able to meet the requirements of high affinity and specificity for measurements of T in body fluids have not been successfully developed by the hybridoma technique [131]. Accordingly, most commercial T-immunoassays rely on polyclonal antibodies. Clearly, the engineering of specific and effective monoclonal antibodies and antibody fragments against testosterone [183] and their production by the phage display technique [184] is of great current interest. From the point of view of analysis, compensation of the cross-reactivity of antibodies and their fragments by applying separation mechanisms capable of resolving the cross-reacting compounds, is of equal interest.

2.6.2 Immunoaffinity solid-phase extraction

Ideally, a sample preparation technique should be solvent-free, simple, inexpensive, efficient, selective, and compatible with a wide range of separation methods [170]. Immunoaffinity solid-phase extraction (IA-SPE) meets many of these requirements.

IA-SPE takes place on a support material on which antibody proteins are anchored (immunosorbent, IS) [169]. There are two effective procedures for immobilizing antibodies (and their fragments): 1) covalent binding directly to a chemically activated support material and 2) attachment *via* secondary molecules such as metal ions [56]. Typical reagents for sorbent activation are N,N-carbonyldiimidazole, cyanogen bromide, glutaraldehyde, and N-hydroxysuccinimide [185].

Support materials such as silica [88], activated alumina [186], controlled porous glass beads [84, 89], and agarose [187, 188] and other soft gels [185] have been reported. Porous sorbent materials allow the preparation of immunosorbents with high antigen capture capacity [56]. Non-specific interactions caused by hydrophobic or ionic forces between IS and interfering compounds in the sample should be avoided. The interactions generated by the sorbent itself have been shown to be minimal in agarose and silica-based ISs [189].

Antibody immobilization procedures suitable for preparing effective ISs are of current interest. Optimally, antibodies should be highly oriented on the sorbent material to provide high antigen capture capacity. Traditional antibody immobilization methods, such as binding *via* antibody amine groups provide random orientation, and are inefficient. As discussed below, a number of more sophisticated immobilization methods have been developed.

In our recent review [86], we highlighted some of the more useful immobilization methods from the point of view of IACE (immobilization of antibodies, or their fragments, on the capillary inner wall). These methods rely on the formation of disulfide bonds between the cysteine groups of an antibody (fragment) and functional groups on the capillary wall. Especially useful is the method introduced by Phillips et al. [190], in which antibody Fab' fragments are immobilized in a highly oriented way through the formation of covalent disulfide bridges between free thiol groups in the hinge region of the fragments and the maleimide-activated capillary wall.

Hale [191] introduced an efficient method for antibody immobilization based on the affinity between Co^{2+}-iminodiacetate (IDA)-Sepharose and a histidine tag genetically inserted into the Fc region of an antibody. High capacity of the IS was obtained through optimal orientation of the antibodies. After oxidation of the cobalt to Co^{3+} under mild conditions, an irreversible complex was obtained, resistant to metal chelating agents, detergents, chaotropic agents, and high salt concentrations. After slight modifications, the protocol of Hale was successfully employed by Nevanen et al. [172] in developing a high-throughput enantiomer-specific IA-SPE format based on a recombinant antibody Fab fragment. The high capacity and stability of the IS were again confirmed. Interestingly, not even a wash with 500 mM imidazole could destabilize the bonds between the Fab fragment and oxidized cobalt (whereas an identical wash of Cu^{3+}-IDA-Sepharose resulted in broken bonds between the Fab fragments and oxidized copper) [172].

The IA-SPE procedure comprises the following steps: (i) conditioning of the immunosorbent, (ii) percolation of the sample, (iii) washing, (iv) elution of the target analytes, and (v) regeneration of the immunosorbent [189]. Centrifugation, filtration, dilution, and modification of the pH of the sample may be necessary before the second (ii) step [189]. The maximum sample volume that can be percolated through the immunosorbent without loss of recovery, i.e. breakthrough volume, depends on the capacity of the IS and the affinity of the antibodies towards the analytes [189].

Elution (iv), *i.e.*recovery of the captured analytes, can be performed by 1) lowering the pH of the eluant, 2) introducing a polarity-reducing agent (such as methanol or ethylene glycol), or 3) applying a chaotropic agent, such as thiocyanate, iodide, or chloride, to disrupt the structure of water molecules around the antibody-antigen complex and induce complex dissociation [86]. Unfortunately, chaotropic agents often disturb the tertiary structure of the antibody in an irreversible way [192]. The most popular elution approach is to use an organic modifier, such as MeOH, EtOH, and ACN [188, 189].

Pharmaceutical and biomedical applications of in-line and off-line IA-SPE were reviewed by Delaunay-Bertoncini and Hennion in 2004 [189]. The hyphenation of IA-SPE with HPLC and CE is becoming more popular, as was discussed in section 2.1.4. It needs to be emphasized, however, that hyphenation of IA-SPE

with HPLC is easier than hyphenation with CE, especially when it comes to the analysis of electrically neutral analytes.

The first hyphenated IA-SPE–HPLC application for AASs, describing the determination of 19-nortestosterone in calf urine, was reported in 1988 [193]. The immunosorbent consisted of polyclonal class-specific antibodies randomly immobilized on CNBr-activated agarose. Two related reports, describing the determination of 19-nortestosterone [194] and estradiol in serum [186] by hyphenated IA-SPE-HPLC have appeared, both relying on randomly immobilized polyclonal antibodies.

Recently, Su et al. [188] described the use of off-line multi-target IA-SPE in the determination of three estrogens (estradiol, estrone, and estriol) in urine of pregnant women samples by MEKC. The immunosorbent consisted of whole polyclonal anti-estrogen antibodies immobilized on CNBr-activated Sepharose. Antibody immobilization was based on non-specific binding *via* amino groups resulting in random orientation of the antibodies. Before IA-SPE, the urine samples were centrifuged for 30 min. The supernatant was then loaded onto the IA-SPE column, the column was washed with PBS, and the estrogens were eluted with MeOH / H_2O (80/20, v/v). The eluted fractions were then diluted and analyzed by MEKC. Investigation of the stability of the IS showed that it could be used at least 20 times and stored at +4 °C over three months.

Hitherto, no IA-SPE applications have been reported for testosterone, perhaps due to failure to produce effective anti-testosterone antibodies by the hybridoma technique. Specific and effective monoclonal antibodies or antibody fragments against testosterone are nevertheless highly desired. As discussed in section 2.3.1, commercial immunoassays for T often suffer from cross-reactions with structurally similar steroids, which lead to inaccurate results. Traditional steroid extraction methods, such as SPE and LLE, are not specific either, and do not allow effective separation of T from other hydrophobic components of the sample. Simultaneous use of high-quality T-specific antibodies and a highly effective separation method for T and related steroids, hyphenated with a sensitive detector, should allow a more reliable determination of T in complex matrices.

2.7 Identification of testosterone and related compounds by electrospray ionization mass spectrometry

Use of electrospray ionization mass spectrometry (ESI-MS) for the identification of testosterone and related steroids is of great interest due to its confirmed suitability for hyphenation with capillary electromigration techniques [195], especially capillary electrophoresis [196–198], and the superior selectivity of MS detection.

In electrospray ionization mass spectrometry (ESI-MS) the ions are transferred from solution to the gas phase, either directly by evaporation from small droplets near the Rayleigh limit (ion evaporation model), as suggested by Iribarne and Thomson [199], or through a series of coulombic fissions leading to formation of single gas-phase ions (charge residue model), as described by Dole et al. [200]. According to Kebarle [201], the ion evaporation model is more likely for small ions.

The electrospray is a special kind of electrolytic cell, which provides an electrochemical conversion of ions to electrons. The difference relative to conventional electrolytic cells working in still solutions is that part of the ion transport occurs through the gas phase, where unipolar charged droplets and the consequent gas-phase ions are the charge carriers [202] (Figure 8).

When the concentration of ions of one polarity at the surface of the liquid at the capillary tip reaches the point at which coulombic forces are sufficient to overcome the surface tension of the liquid, droplets enriched in one ion polarity are emitted from the capillary (Figure 8). To maintain the continuous loss of one ion polarity, the formation of opposite charge in the capillary must be neutralized. The charge-balancing process involves electrochemical oxidation/reduction of the components of the metal capillary and/or one or more species in the solution. This leads to an electron flow from (negative ion mode) or to (positive ion mode) the high-voltage supply.

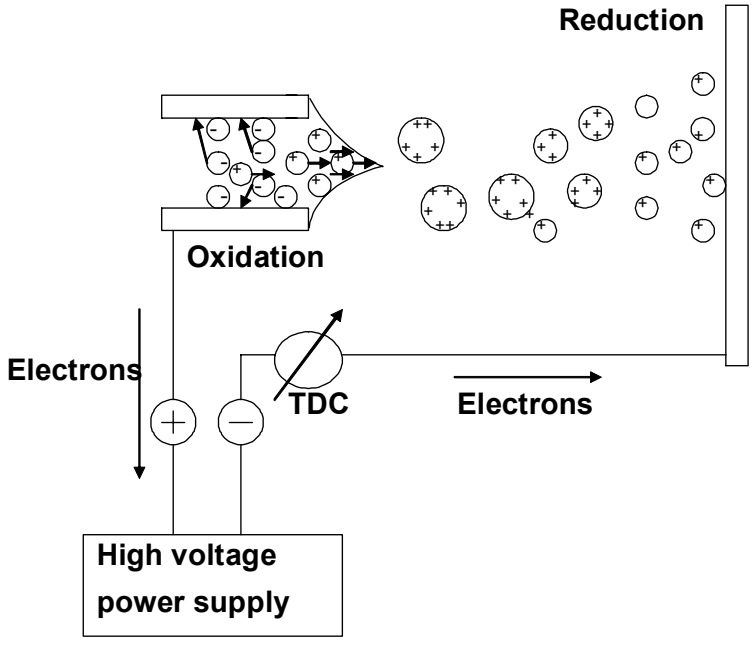

Figure 8. Formation of gas-phase ions in ESI-MS in positive ion mode. TDC is the total droplet current. Modified from Ref. 201.

The most common way to hyphenate capillary electromigration techniques, including (PF)-MEKC, with ESI-MS is *via* sheath liquid [195]. The primary function of the sheath liquid is to provide electrical contact between the two instruments. An unfortunate drawback of this interfacing is the high flow rate of the sheath liquid, which results in diluted analyte bands and decreased sensitivity [195]. Sheathless CE-ESI-MS has attracted growing attention during recent years, but standardized designs and protocols are currently lacking [203].

Measured in terms of the degree to which fragmentation of the analytes occurs, ESI is a relatively soft ionization technique. ESI of the analytes will be adversely affected by the presence of other ions if these suppress analyte ionization [201]. Other ionization techniques, such as APCI (atmospheric pressure chemical ionization) [28, 42] and APPI (atmospheric pressure photoionization) [204], have been introduced as alternatives to ESI, but for T-related steroids, ESI offers slightly better sensitivity [205, 206].

Identification and especially the quantification of T and related steroids by ESI-MS are highly challenging. ESI-MS behavior of the analytes must be known when reliable quantification is desired. ESI-MS analysis of T-related steroids as their glucuronide conjugates is not simple, especially when it comes to T and E. The glucuronide bond to E is significantly more labile than that to T [163]. According to Kuuranne et al. [207], because the molecular strain in epitestosterone glucuronide is clearly greater than that in testosterone glucuronide, the precursor ion $[M+H]^+$ of epitestosterone glucuronide in ESI-MS is significantly less stable than that of testosterone glucuronide. Furthermore, steroid glucuronides have a strong tendency to form ammonium adducts, but the extent of adduct formation may vary significantly [163]. After exhaustive HPLC–ESI-MS studies, Bowers and Sanaullah [163] concluded that epitestosterone glucuronide forms ammonium adducts to a much greater extent than testosterone glucuronide.

Determination of free T and related steroids is less complicated than the determination of their glucuronide conjugates. Ma and Kim [205] investigated the ESI-MS behavior of 29 steroids, including T and E, by HPLC–ESI-MS. In positive ion mode, when the mobile phase consisted of MeOH / 0.1% acetic acid in water (80/20, v/v), both T and E produced their most stable and intense ions $[M + H]^+$, 100% and 100%, and $[M + Na]^+$, 58% and 68%, respectively. Even better sensitivity (between 5 and 15 pg) was obtained by single ion monitoring for $[M + Na]^+$ when the presence of protons was minimized.

Later, Leinonen at al. [206] studied the HPLC–ESI-MS behavior of a different set of steroids, including fluoxymesterone, metandienone, and MT in positive ion mode. Metandienone and MT produced $[M + H]^+$ as their most intense ion (100%), whereas F had a stronger tendence to form $[M + NH_4]^+$. All steroids could be detected within 0.1–2.0 ng/mL. The method showed good linearity ($r^2 = 0.995$) up to 250 ng/mL.

Until now, ESI-MS has not been hyphenated with any capillary electromigration technique for the identification of T and related steroids. Thus, especially the use of PF-MEKC (discussed in section 2.1.2) interfaced with ESI-MS is of great interest.

3. Aims of the study

The overall objective of the study was to analyze testosterone and related compounds by capillary electromigration techniques making use of non-specific and specific interactions between steroids and additives, either mobile or immobilized. New methods for the determination of steroids were developed. Furthermore, new sample pretreatment procedures, suitable for the specific determination of testosterone in urine samples, were explored.

Specifically the main aims were to:

- develop efficient analytical methods for testosterone and structurally similar steroids based on micellar electrokinetic chromatography (**I–IV**) and immunoaffinity capillary electrophoresis (**III**)
- optimize the identification of steroids by UV spectrophotometry (**I–III, V**) and electrospray ionization mass spectrometry (**I, IV**)
- develop a new immunoaffinity solid-phase extraction procedure for the determination of testosterone in urine samples (**III**)
- study the interactions between steroids and plasma proteins by affinity capillary electrophoresis (**V**).

4. Experimental

4.1 Chemicals and materials

The chemicals and materials used in the study are listed in Table 4. The purpose of each item is shortly noted. More detailed information is given in the original publications I–V.

Table 4. Chemicals and materials used in the study.

Compound / material	Manufacturer / Supplier	Notes	Publication
Acetic acid	Merck	For pH adjustment	I
Androstenedione	1*, Steraloids	Standard	I–V
Ammonia	Riedel-de Haën	25%, for pH adjustment	II
Ammonium acetate	Fluka	Electrolyte salt	I–IV
Argon	Aga	Collision gas	I, IV
BCA Protein Assay Reagent Kit	Pierce	For colorimetric detection of total protein	III
Bovine serum albumin, essentially globulin- and fatty acid-free	Sigma	Standard	V
Bovine serum albumin, essentially globulin-free	Sigma	Standard	V
Chelating Sepharose Fast Flow	Amersham-Pharmacia	Sorbent	III
Cobalt chloride hexahydrate ($CoCl_2 \times 6H_20$)	Merck	For oriented immobilization of Fab fragments via histidine tag	III
Diethanol amine magnesium chloride	Reagena	ELISA reagent	
Diethyl ether	Rathburn	Solvent	II
17-Epimetandienone	1*	Standard	I
Epitestosterone	1*	Standard	II–V
Ethylenediamine tetraacetic acid (EDTA)	Sigma	Chelator	III
Ethanol	Primalco	Solvent, EOF marker	
Fluoxymesterone	Steraloids	Standard	I–III, V

Fused silica capillary	Composite Metal Services, Polymicro Technologies	i.d. 50 µm, o.d. 375 µm i.d. 50–51 µm, o.d. 360 µm	I, III, IV II, V
GelCode® Blue Stain Reagent	Pierce	For protein staining	III
goat anti-human Fab_2 alkaline phosphatase	Rockland	ELISA reagent	
β-Glucuronidase from *E. coli*	Sigma	Hydrolyzing reagent, for deconjugation of glucuronide conjugates	III
Heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)cyclomaltoheptaose (HDAS)	2*	> 98%, electrolyte additive	II
Heptakis(2,3-dihydroxy-6-*O*-sulfo)cyclomaltoheptaose (HS)	2*	> 97%, electrolyte additive	II
Heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)cyclomaltoheptaose (HDMS)	2*	> 98%, electrolyte additive	II
Human serum albumin, essentially globulin- and fatty acid-free	Sigma	Standard	V
Human serum albumin, essentially globulin-free	Sigma	Standard	V
4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)	Sigma	Electrolyte salt	III
Hydrogen peroxide	Riedel-de Haën	30%, oxidizing reagent	III
Metandienone	1*	Standard	I
Methanol	Rathburn	Solvent, EOF marker	
Methyltestosterone	1*, Steraloids	Internal standard, standard	I–V
MF filter	Millipore	0.45 µm, biologically inert mixture of cellulose acetate and cellulose nitrate	I–V
Nitrogen	Aga	99.997%, nebulizing gas, drying gas	I, II, IV
p-Nitrophenylphosphate disodium salt	Sigma	ELISA reagent	
Octakis(2,3-di-*O*-acetyl-6-*O*-sulfo)cyclomaltooctaose (ODAS)	2*	> 98%, electrolyte additive	II
Octakis(2,3-dihydroxy-6-*O*-sulfo)cyclomaltoheptaose (OS)	2*	> 99%, electrolyte additive	II
Octakis(2,3-di-*O*-methyl-6-*O*-sulfo)cyclomaltooctaose (ODMS)	2*	> 98%, electrolyte additive, containing 1% by weight sodium sulfate	II–IV

Polytetrafluoroethylene (PTFE) frit	Teaninich Industrial Estate Alness	For preparation of the IA-SPE cartridge	III
Polytetrafluoroethylene (PTFE) tubing	Zeus Industrial products	i.d. 406 µm, for preparation of the IA-SPE cartridge	III
2-Propanol	Rathburn	Solvent	IV
Sodium chloride	Riedel-de Haën	For phosphate-buffered saline (PBS)	III
Sodium cholate	Sigma	Surfactant	
Sodium dodecyl sulfate	Sigma	Surfactant	I–IV
Sodium hydroxide	Merck	For capillary conditioning	II
Sodium nitrite	Sigma	Preservative	III
Sodium phosphate dibasic ($Na_2HPO_4 \times 2\ H_2O$)	Merck	For phosphate-buffered saline (PBS)	III
Sodium phosphate monobasic ($NaH_2PO_4 \times 2\ H_2O$)	J. T. Baker	For phosphate-buffered saline (PBS)	III
Strata™ X	Phenomenex	SPE cartridges, 30 and 100 mg	II, III
Taurocholic acid, sodium salt	Calbiochem	Surfactant	I–IV
Tauroglycocholic acid, sodium salt	Merck	> 65%, surfactant	
Testosterone	Steraloids	Standard	I–V
Tris[hydroxymethyl]aminomethane	Sigma	Electrolyte salt	V
Testosterone-3-(O-carboxymethyl)oxime-BSA	Sigma	ELISA reagent	
Tris[hydroxymethyl]aminomethane hydrochloride	Fluka, Sigma	Electrolyte salt	III, V
Tygon tubing	Ismatec	i.d. 380 µm, for IA-SPE	III
Water	Millipore	Milli-Q	I–V

1* A gift from United Laboratories Ltd, Helsinki, Finland (a doping laboratory accredited by WADA (World Anti-Doping Agency))

2* A gift from Prof. Guyla Vigh's group, Department of Chemistry, Texas A&M University, College Station, USA

4.2 Instruments

All capillary electromigration analyses in studies I–V were performed with a P/ACE MDQ instrument (32 Karat Software, version 8.0) equipped with a photodiode array detector (Beckman Coulter Instruments, Fullerton, CA, USA).

The mass spectrometric experiments in studies I and IV were performed with a Quattro II triple quadrupole instrument equipped with an ESI interface (Micromass, Manchester, UK).

ELISA measurements were performed with a Varioskan instrument (Thermo Electron).

All pH measurements were performed with a Denver model 20 pH meter with combination electrode (Denver Instrument Company, CO, USA) calibrated with pH 4.00 (±0.01), 7.00 (±0.01), and 10.00 (±0.01) commercial buffers (Reagecon, Shannon, Ireland).

Conductivity measurements were performed using a Denver model 20 pH/ conductivity meter with an epoxy-body conductivity cell of 0.1 cm^{-1} (Denver Instrument Co., USA). The sensor was calibrated with a conductivity standard solution (84 µS/cm at +25°C) (Reagecon, Shannon, Ireland).

4.3 Methods

The methods used in the original studies (I–V) of this thesis are listed in Table 5.

Table 5. Methods applied in the study.

Method	Publication
PF-MEKC	I–IV
UV spectrophotometry	I–V
CE (CZE)	II, V
Conductivity measurements	II
Sweeping	III, IV
Direct infusion ESI-MS	I, IV
PF-MEKC–ESI-MS	I, IV
Enzymatic hydrolysis with *β*-glucuronidase	III
SPE (non-specific)	II, III
ELISA	III

Immobilization of the Fab fragments	III
BCA Protein Assay	III
SDS-PAGE	III
Immunoaffinity solid-phase extraction (IA-SPE)	III
IACE (IA-SPE–PF-MEKC)	III
PF-ACE	V

Some of the methods and protocols are briefly described in the following sections. More detailed information, including optimization of the methods, is given in the original publications I–V.

4.3.1 PF-MEKC and UV spectrophotometry

The conditions used in the PF-MEKC–UV experiments with T and related steroids in studies I–IV are listed in Table 6. Before the analyses, the fused-silica capillaries were conditioned by rinsing at 20 psi (138 kPa) with 0.1 M NaOH, deionized water, and BGE for 10–15 min. The steroids were detected at 247 nM. Between analyses, the capillary was rinsed for 3–5 min with the background electrolyte at 20–30 psi (I, II, IV), or for 1 min with the pseudostationary phase and for 4 min with the BGE at 25 psi (172 kPa) (III). In all experiments, the total length of the capillary was 80 cm and the effective length 70 cm. All injections were performed by pressure.

Table 6. Analysis conditions in the PF-MEKC–UV experiments.

Publication	BGE	Pseudostationary phase(s), approximate plug length	Sample matrix and volume (nL)	V (kV)	Temp. (°C)	Analytes
I	20 mM ammonium acetate, pH 9.5	29.3 mM SDS / 1.1 mM sodium taurocholate / 6.7% (v/v) MeOH in 18.7 mM ammonium acetate, 3.5 cm	BGE / EtOH (50/50, v/v) 4 nL	+25	+22	A, F, T, MT, metandienone, 17-epimetandienone
II	15 mM ammonium acetate, pH 9.5	29.5 mM SDS / 1.5 mM sodium taurocholate in 15.6 mM ammonium acetate, and 29.5 mM SDS / 20 mg/mL ODMS in 14.6 mM ammonium acetate, 4.2 cm in total	BGE / EtOH (80/20, v/v), 8 nL	+30	+30	A, F, T, MT, E
III	30 mM ammonium acetate, pH 9.5	29.5 mM SDS / 1.5 mM sodium taurocholate in 30 mM ammonium acetate, and 29.5 mM SDS / 20 mg/mL ODMS in 30 mM ammonium acetate, 4.2 cm in total	BGE / EtOH (80/20, v/v), 80 nL	+30,	+30	A, F, T, MT, E
IV	20 mM ammonium acetate, pH 9.5	29.3 mM SDS / 1.5 mM sodium taurocholate in 20 mM ammonium acetate, 2.8 cm	BGE / EtOH (80/20, v/v) 37 nL	+30, plus 0.1 psi back pressure	+25, +30	A, T, MT, E

4.3.2 Capillary (zone) electrophoresis

The migration of taurocholic acid monomers and octakis(2,3-di-*O*-methyl-6-*O*-sulfo)cyclomaltooctaose (ODMS) was investigated in study II by CE with use of 15 mM ammonium acetate (pH 9.5) as the background electrolyte. The operating voltage was +30 kV, the capillary temperature +30 °C, the detection wavelength 195 nM, and the capillary length L_{det}/L_{tot} 70/80 cm. The EOF marker was ethanol.

In study V, the migration of steroids in the absence of albumin proteins was investigated by CE with 20 mM Tris-HCl (pH 7.8) as the BGE. The operating voltage was +20 kV and the capillary temperature +37 °C.

4.3.3 Conductivity measurements

In study II, the CMC of taurocholate was estimated by conductivity measurements. When the conductivity of a series of solutions with increasing concentrations of surfactant is measured, the specific conductivity vs. surfactant concentration plot shows two straight lines with different slopes. Above the CMC, the slope is lower [208]. The samples containing 0–4 mM taurocholic acid in 15 mM ammonium acetate (pH 9.5) were thermostated at +30 °C.

4.3.4 Direct infusion ESI-MS

In studies I and IV, ionization of steroids was studied by ESI-MS in positive ionization mode. The samples were introduced with a 1.0-mL syringe (Hamilton, Reno, NV, USA) by using a microsyringe pump (Harvard Apparatus, South Natick, MA, USA). The scan range; optimized flow rate of the drying gas (N_2); flow rate of the nebulizing gas (N_2); capillary, counter electrode (HV) lens, and cone voltages; the source temperature, collision energy and the pressure of the collision gas (Ar) applied in studies I and IV, as well as the analysis conditions, are compiled in Table 7.

Table 7. Instrumental parameters and conditions applied in the direct infusion ESI-MS experiments of studies I and IV.

Parameter	I	IV
scan range in MS mode (m/z,amu)	50–800	50–650
scan range in MS/MS mode (m/z, amu)	20– ($[M + H]^+$ + 20)	20–320
flow rate of sample solution (μL/min)	5	10
flow rate of the drying gas (L/h)	250	250
flow rate of the nebulizing gas (L/h)	10–20	15
capillary (kV), counter electrode (HV) lens (kV), and cone voltages (V)	+4.0, +0.5, +40 V	+3.5, +0.5, +40 V
source temperature (°C)	+ 70	+ 70
collision energy (eV) in MS/MS	20–30	25
pressure of the collision gas in MS/MS (μbar)	1.7	1.7
sample matrix	MeOH / 20 mM ammonium acetate-acetic acid (50/50/0.1%, v/v/v)	MeOH / 20 mM ammonium acetate-acetic acid (75/25/0.1%, v/v/v)
steroid concentration (μg/mL)	5–10	7.5

4.3.5 PF-MEKC–ESI-MS

PF-MEKC–ESI-MS of steroids was investigated in studies I and IV. The CE and MS instruments were connected through a commercial interface with coaxial sheath flow. The sheath liquid was introduced with a 1.0-mL (I) or 0.5-mL (II) syringe (Hamilton, Reno, NV, USA) by using a microsyringe pump (Harvard Apparatus, South Natick, MA, USA). The parameters (see section 4.3.2) and conditions applied in the PF-MEKC–ESI-MS studies are listed in Table 8. The HV lenses were removed from the ionization chamber as suggested by Vuorensola et al. [196]. Temperature control of the whole separation capillary was not possible. No drying gas was used.

Table 8. Parameters and conditions applied in the PF-MEKC–ESI-MS experiments of studies I and IV.

Parameter	I	IV
separation voltage (kV), back pressure (psi)	+20, 0.5	+30, 0.1
background electrolyte (BGE)	20 mM ammonium acetate (pH 9.5)	20 mM ammonium acetate (pH 9.5)
pseudostationary phase, approximate plug length	29.3 mM SDS / 1.1 mM sodium taurocholate / 6.7% MeOH, 3.5 cm	29.5 mM SDS / 1.5 mM sodium taurocholate, 2.8 cm
selected ions (m/z amu) in SIM	337.4, 289.3, 287.3	303.2, 289.2, 287.2
application of TIC of the selected channels	no	yes
flow rate of the sheath liquid (μL/min)	10	5
sheath liquid composition	MeOH / BGE-acetic acid (50/50/0.1%, v/v/v)	MeOH / BGE-acetic acid (70/30/0.1%, v/v/v)
flow rate of the nebulizing gas (L/h)	50	42.5
capillary voltage (kV) and cone voltages (V)	+3.5, +40	+3.5, +40
source temperature (°C)	70	70
dwell time (s)	0.08	0.08
interchannel delay (s)	0.01	0.01

4.3.6 Enzymatic hydrolysis of glucuronide conjugates

Enzymatic hydrolysis of glucuronide conjugates of T was performed in study III. Small aliquots (0.8 mL) of male urine samples were buffered with 67 mM phosphate (pH 7.0) (85/15, v/v). Lyophilized *β*-glucuronidase from *E. coli* (≥ 1000 U) was dissolved in 1 mL of 4 mM phosphate buffer (pH 6.8). An aliquot of 50 U of *β*-glucuronidase per mL of urine was added to the samples. Enzymatic hydrolysis was performed at +60 °C (water bath) for 1 h. After hydrolysis, the samples were cooled down to room temperature. An aliquot of 800 μL per sample was analyzed by the off-line IA-SPE and PF-MEKC as described in sections 4.3.1 and 4.3.9 (Table 6).

4.3.7 Non-specific SPE

Analysis of steroids from spiked urine samples by PF-MEKC was investigated in study II. To 5-mL aliquots of male urine was added 5 µL of 100 mg/L T, A, and E (leading to a final concentration of 100 ng/mL for each). The spiked urines were purified in preconditioned (with 600 µL of methanol) and equilibrated (with 600 µL of deionized water) Strata™ X (30 mg) cartridges. The impurities were washed out with 1.2 mL of 2% NH_4OH, and the cartridges were dried for approximately 30 s at full vacuum. The elutions were carried out with 700 µL of diethyl ether, and the eluates were evaporated with a gentle stream of pure nitrogen (99.997%) at room temperature. Before the PF-MEKC analyses, the residues were dissolved in 15 mM ammonium acetate (pH 9.5) / ethanol (80:20, v/v). To discover the matrix effect, steroids were extracted from male urine as described above. The steroid-free urine percolating through the cartridges was collected.

In study III, steroid-free female urine was used as matrix for spiked urine samples in order to minimize the presence of natural T. Estrogens and possible androgens at trace levels were removed from the female urine by SPE performed with Strata™ X (100 mg) cartridges. The cartridges were preconditioned with 1.2 mL of methanol and equilibrated with 1.2 mL of deionized water before SPE. The steroid-free urine percolating through the cartridges was collected.

4.3.8 Determation of affinity between testosterone and the recombinant anti-testosterone Fab fragment by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to determine the affinity between testosterone (actually testosterone-3-(O-carboxymethyl)oxime-BSA) and a recombinant anti-testosterone antibody Fab fragment, recently introduced by Lu et al. [184]. The Fab fragment (Fab220) was donated by Prof. Takkinen's group. A 96-well plate was coated with 500 ng/well of testosterone-3-(O-carboxymethyl)oxime-BSA in bicarbonate buffer (pH 9.8) for 18 h at +4 ºC. The plate was then washed three times with 20 mM sodium phosphate / 150 mM NaCl (pH 7.4) (PBS). The wells were blocked with 200 µL of 0.5% BSA-PBS for 1 h at room temperature, and washed as described above. Fab220 samples were prepared in 0.5% BSA-PBS. An aliquot of 100 µL of Fab220 (100 µg/mL) and a series of Fab220 dilutions (1:100, 1:200, 1:500, 1:1000 and

1:1500) were pipetted on wells as duplicates (100 µL/well). The samples were incubated at room temperature for 2.5 h, and washed with PBS. Aliquots of 100 µL of goat anti-human Fab_2 alkaline phosphatase, diluted 1:1000 in 0.5% BSA-PBS, were pipetted into Fab220 wells. After 1 hour incubation at room temperature and a wash with PBS, the substrate (100 µL of 2 mg/mL of *p*-nitrophenylphosphate) was pipetted into each well. The absorbance was measured at 405 nM after 14 and 20 min. The K_d value was derived from the linear part of the curve (response against Fab concentration).

4.3.9 Preparation of anti-T immunosorbent

An anti-T immunosorbent was prepared in study III. A recombinant anti-testosterone Fab fragment (Fab220) was immobilized on Chelating Sepharose Fast Flow matrix *via* the six-histidine tag as described by Nevanen et al. [172]. Chelating Sepharose (5 mL) was loaded with 12.5 mL of 200 mM $CoCl_2$ in water and incubated in a rotator for 2.5 hours at room temperature. An aliquot of 1 mL of the Co^{2+}–iminodiacetic acid–Sepharose was then washed with deionized water. An aliquot of 8.0 mL of Fab fragments (3.2 mg/mL) in 20 mM sodium phosphate / 150 mM NaCl (PBS, pH 7.4) was poured on the gel and incubated in a rotator at +4 °C for 16 h. The supernatant, as well as the following washing and reagent solutions, were then collected for protein determinations. The gel was washed with deionized water, and the cobalt was oxidized with 2 mL of 0.03% H_2O_2 for 3.5 hours at room temperature. After this, the gel was washed with deionized water and 50 mM EDTA. After a final wash with deionized water, the immunosorbent was equilibrated with PBS (pH 7.4), and NaN_3 was added as preservative to a final concentration of 0.02% (v/v). The immunosorbent was stored at + 4 °C before use. The supernatant, washing, and reagent solutions that had been in contact with the immunosorbent were analyzed to determine the concentration of the bound Fab fragments on the immunosorbent. Determinations were made by BCA Protein Assay, according to the manufacturer's instructions (using BSA and the purified anti-testosterone Fab fragment as standards), by UV spectrophotometer measurements at wavelengths 260, 280, and 320 nM, and by SDS-PAGE.

4.3.10 Immunoaffinity solid-phase extraction

In study III, a microscale anti-T immunosorbent column was set up by filling a Tygon® tube (i.d. 380 µM, length 6.0 cm) with 5.0 µL of the immunosorbent in PBS (pH 7.4). The immunosorbent was held in the column by a small PTFE frit. IA-SPEs were performed at +23 °C. The immunosorbent was washed with 100 µL of 50% ethanol in 30 mM NH_4OH (pH 9.5) and 100µL of 25 mM NH_4OH (pH 7.0) before use (preliminary elution) to remove the free light chains of the Fab fragment. The samples were manually injected to the column with a gas-tight 1000-µL Hamilton syringe (Hamilton Bonaduz, Switzerland). After injection, the immunosorbent was rinsed with 100 µL of 25 mM NH_4OH (pH 7.0). Subsequently, T was eluted with 70 µL of 50% ethanol in 30 mM NH_4OH (pH 9.5). The immunosorbent was conditioned with 100 µL of 25 mM NH_4OH (pH 7.0) before each extraction. In each stage, the fractions were collected directly in the CE instrument sample vials, diluted with BGE, and spiked with MT and A leading to a final concentration of 0.5 mg/L of external standards and 20% of ethanol. The samples were analyzed by the PF-MEKC method described in section 4.3.1 (Table 6).

4.3.11 Hyphenated IA-SPE–PF-MEKC

In-capillary IA-SPE–PF-MEKC analysis of testosterone was investigated in study III. Immunosorbent (0.5 µL), diluted in 20 mM Hepes (pH 7.4), was integrated to a commercial capillary cartridge. The background electrolyte was 20 mM Hepes (pH 7.4). The eluant consisted of 50% ethanol in 20 mM Hepes (pH 7.4), and the pseudostationary phase of a ~ 13 cm long plug of 50 mM SDS in 20 mM Hepes (pH 7.4). The capillary containing the immunosorbent was equilibrated with the BGE before sample injection. The sample was hydrodynamically introduced to the capillary at 6.89 kPa from the outlet side for 30 s. Subsequently, the capillary was rinsed with the BGE at 34.5 kPa for 10 min. The eluant plug was injected at 68.9 kPa for 0.1 min followed by a plug of the BGE at 68.9 kPa for 0.1 min. Finally, the pseudostationary phase was injected at 68.9 kPa for 0.45 min before application of +25 kV voltage. All the solutions were injected to the capillary from the outlet side.

4.3.12 Affinity measurements by PF-ACE

In study V, the affinity between steroids and albumins (HSA, BSA) was investigated by partial filling ACE. The analysis conditions are compiled in Table 9.

Table 9. Analysis conditions applied in the PF-ACE studies (publication V).

Background electrolyte, pH	20 mM Tris-HCl, pH 7.8
Capillary dimensions	L_{det}/L_{tot} 70/80 cm, i.d. 50 µm
Temperature	+ 37 °C
Voltage	+ 20 kV (250 V/cm)
Current	+8.5 µA
Detection wavelengths	214 and 247 nM
HSA concentration	95 µm
BSA concentration	190 µm
Molar ratio between HSA and a steroid	5–25
Molar ratio between BSA and a steroid	10–50

The calculations of association constants between albumins (HSA and BSA) and electrically neutral steroids were based on a series of PF-ACE experiments, in which the total molar amount of the protein (n) introduced into the capillary was varied (by applying a sequence of injections for 0, 20, 40, 60, 80 and 100 s at 3447 Pa), and the change in the migration rate of the steroids related to the amount of protein was monitored. The association constants, K_b, were determined from a plot of (t_a-t_{eof}) against n using the following equation:

$$K_b = \frac{1}{K_d} = \frac{\pi r^2 L_{\text{det}}}{t_{0,eof}} \cdot \frac{d(t_a - t_{eof})}{dn} \qquad (10)$$

where r is the inner radius of the capillary, L_{det} the effective length of the capillary, t_a the migration time of the steroid, t_{eof} the migration time of a neutral electroosmotic flow marker, $t_{0,eof}$ the migration time of a neutral electroosmotic flow marker in the absence of protein, and n the total molar amount of protein.

Eq. 10 was modified from Eq. 11 presented by Nilsson et al. [76]

$$K_b = \frac{1}{K_d} = \frac{\pi r^2 L_{\text{det}}}{t_{0,\text{det}}} \cdot \frac{d(t_{\text{det}} - t_{0,\text{det}})}{dn} \qquad (11)$$

where $t_{0,\text{det}}$ is the time needed for the analyte to reach the detector in the absence of protein, and (t_{det}-$t_{0,\text{det}}$) is the difference between the times needed for the analyte to reach the detector in the presence and absence of protein.

According to Nilsson et al. [76], the migration of a ligand in PF-ACE depends on the absolute amount of the injected protein in a linear way. Furthermore, the migration is independent of both protein mobility and protein plug length. Factors such as electric field strength and EOF are irrelevant if kept constant during the analyses.

In our PF-ACE studies, however, EOF was needed to transfer the electrically neutral steroids to the detector. Thus, we could not use Eq. 11 as such. In our studies, the variation of EOF was compensated with use of a neutral, non-interactive internal standard (EtOH). Instead of measuring the difference between the times needed for the analyte to reach the detector in the presence and absence of albumin, we measured the difference between the migration times of the analyte and EtOH in each run. The electroosmotic flow in the capillary filled with neat electrolyte solution had to be controlled to keep $t_{0,\text{eof}}$ constant.

5. Results and discussion

The use of non-specific and specific interactions in the analysis of steroids by capillary electromigration techniques was investigated in this work. New PF-MEKC methods, relying on non-specific interactions between steroids, dynamic micelles, and cyclodextrins, were developed for quantitative determination of testosterone and related steroid standards. Steroid analyses by PF-MEKC–UV, direct infusion ESI-MS, and PF-MEKC–ESI-MS were investigated. New immunoaffinity SPE applications, relying on specific interactions, either in-capillary or off-line, between testosterone and a recombinant anti-testosterone Fab fragment, were tested for the determination of testosterone in male urine samples by PF-MEKC. Finally, interactions between steroids and albumins were investigated by PF-ACE. The results of the investigations are discussed below.

5.1 Analyses of testosterone and related compounds by PF-MEKC–UV

The separation of testosterone and structurally related steroids by MEKC was the focus of the first part of the study. The partial filling (PF) approach was utilized from the very beginning to allow both UV and ESI-MS detection.

Ammonium acetate was selected as the electrolyte salt in view of its volatility and buffering capacity in the pH range (alkaline) of interest. A relatively strong EOF was desired to obtain an efficient separation of the neutral steroids from the anionic surfactants and micelles.

In our first experiments (I), the separation power of a pseudostationary phase consisting of SDS in 20 mM ammonium acetate (pH 9.5) was investigated by PF-MEKC where the concentration of SDS was varied between 25 and 50 mM and the plug length of the pseudostationary phase between 3 and 35 mM (corresponding to 0.4–5.0% of the effective capillary length). SDS is a surfactant possessing hydrogen-bond basicity (though lower than that of water) and moderate polarizability (which is determined by the difference in capacities of the BGE and micellar phase to interact with n- and π-electrons of the analyte) [209]. In our study, SDS alone did not provide separation of the structurally

similar steroids (fluoxymesterone, androstenedione, metandienone, testosterone, methyltestosterone, and 17-epimetandienone), which all have the potential to act as hydrogen-bond acceptors owing to one or several carbonyl group(s) and, except androstenedione, hydrogen-bond donors owing the one or several hydroxyl groups (see Figure 5 in section 2.2). The result was expected since earlier reports had described the insufficient separation power of SDS for structurally related corticosteroids [14, 15, 16]. Separation of 10 corticosteroids in 25 mM SDS / 25 mM borate buffer (pH 9.5) has been reported [210], but non-volatile borate is not an optimal electrolyte for MS detection.

We continued the PF-MEKC–UV analyses with more complex pseudostationary phases consisting of SDS and different bile salts, namely SDS–sodium cholate, SDS–sodium tauroglycocholate, and SDS–sodium taurocholate. Several different ratios of the SDS and bile acid concentrations were tested, as well as plug lengths (28–42 mM) and the presence (1–10%, v/v) of organic additives (ACN, MeOH). A pseudostationary phase consisting of SDS, sodium taurocholate, and MeOH showed efficient resolving capacity for F, A, metandienone, T, MT, and 17-epimetandienone. The best resolution was obtained with a 35-mm plug comprising 29.3 mM SDS / 1.1 mM sodium taurocholate / 6.7% (v/v) MeOH in BGE (I). The PF-MEKC–UV data of study I is compiled in Table 10.

Table 10. PF-MEKC–UV data of studies I–IV, including limits of detection (S/N = 3), absolute RSD% values for peak areas and migration times, and linearity for F, A, metandienone, T, MT, 17-epimetandienone, and E.

Study		Analyte						
		F	**A**	**metandienone**	**T**	**MT (i.s.)**	**17-epimetandienone**	**E**
I	LOD (μg/L)	45	39	90	40	59	45–90	-
I	RSD% (peak area)	14	12	16	11	15	13	-
I	RSD% (migration time)	0.80	0.79	0.81	0.80	0.80	0.76	-
I	Linearity (r^2)	0.994	0.999	0.999	0.999	-	0.998	-
II	LOD (μg/L)	160	100	-	73	80	-	85
II	RSD% (peak area)	4.9	6.2	-	5.7	3.6	-	5.3
II	RSD% (migration time)	1.0	0.94	-	0.84	0.81	-	0.80
II	Linearity (r^2)	0.998	0.997	-	0.999	-	-	0.999
III	LOD (μg/L)	28	9	-	18	21	-	22
III	RSD% (peak area)	3.8	2.0	-	2.3	1.9	-	2.4
III	RSD% (migration time)	0.6	0.6	-	0.7	0.7	-	0.7
III	Linearity (r^2)	0.999	0.999	-	0.999	0.999	-	0.999
IV	LOD (μg/L)	-	65	-	97	72	-	85
IV	RSD% (peak area)	-	2.2	-	1.4	2.6	-	1.4
IV	RSD% (migration time)	-	0.6	-	0.5	0.5	-	0.5
IV	Linearity (r^2)	-	0.998	-	0.999	0.998	-	0.997

The method developed in study I was highly linear (except for F) and sensitive in terms of LODs in comparison with results presented in earlier capillary electromigration reports (from 100 [22] to 115 μg/L [20] for testosterone). However, the absolute RSD% values for peak areas were relatively large (11–16%).

In the following study (II), we focused on optimization of the separation of F, A, T, MT (i.s.), and E. Encouraged by the results obtained in study I, we first applied the developed method with slight variations. A good resolution was obtained with a 4.2-cm plug of 29.5 mM SDS / 1.5 mM sodium taurocholate in 15.6 mM ammonium acetate with 15 mM ammonium acetate (pH 9.5) used as the BGE. The presence of MeOH or EtOH in the pseudostationary phase solution did not improve the resolution. The separation was further enhanced by raising the temperature and voltage from +22 °C to +30 °C, and from +25 kV to +30 kV. However, this method too, suffered from relatively poor repeatability. The absolute RSD values for migration times ranged from 1.2 to 4.6%, and those for peak areas from 12.7 to 15.4%. Next we investigated the effect of using different single-isomer sulfated cyclodextrins (HDMS, HS, HDAS, ODMS, OS, HDMS, Table 4) in place of sodium taurocholate in the pseudostationary phase. Baseline separation for F, A, T, and E was obtained with a 4.2-cm plug comprising 29.5 mM SDS / 20 mg/mL ODMS in 14.6 mM ammonium acetate (pH 9.5). This method provided better repeatability: the RSDs of the migration times were close to 0.9% and those of the peak areas between 5.1 and 6.6%. The explanation of this good result could be the more efficient removal of ODMS vs. taurocholate from the capillary between runs owing to weaker interactions between ODMS and the capillary inner wall as compared with those between sodium taurocholate and the inner wall.

Finally, in study II, we investigated the PF-MEKC analysis of F, A, T, MT, and E utilizing the two optimized pseudostationary phases sequentally. Several different combinations were tested (described in detail in publication II), and the best results in regard to resolution were obtained by sequential injection of the SDS-ODMS and SDS-sodium taurocholate solutions at equal plug lengths (2.1 cm each) (Figure 9). RSD values for peak areas and migration times were close to values obtained with the SDS-ODMS mode. The results of the method optimized in study II are listed in Table 10 above. The sequential PF-MEKC proved to be less sensitive than the method optimized in study I, perhaps because presence of plentiful inorganic ions, including sodium and sulfate, deriving from ODMS.

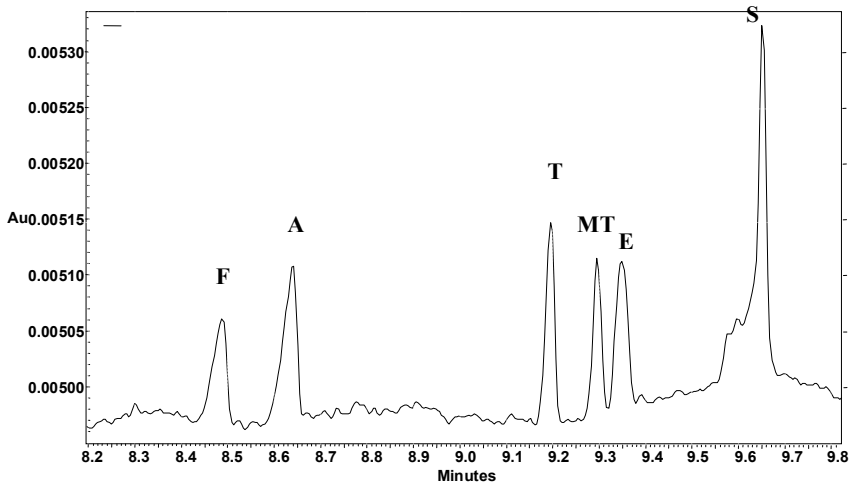

Figure 9. Separation of F, A, T, MT, and E (5 mg/L per each) by the sequential PF-MEKC method (study II). Sample volume 8 nL. S is a system peak. Detection at 247 nM. Detailed analysis conditions in Table 6 (page 64).

The separation mechanism of the sequential PF-MEKC mode was carefully investigated. First, the function of sodium taurocholate was examined by estimating the CMC of taurocholate with conductivity measurements (Figure 10).

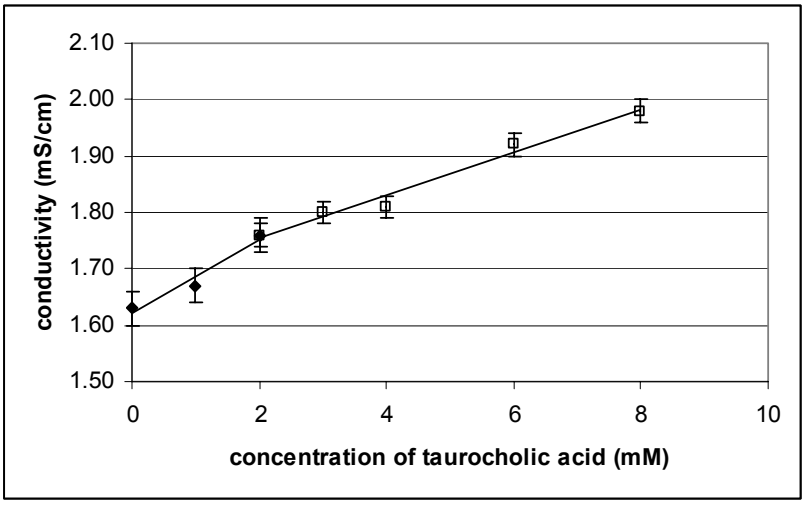

Figure 10. CMC of sodium taurocholate in 15 mM ammonium acetate (pH 9.5).

The CMC of taurocholate in 15 mM ammonium acetate (pH 9.5) at +30 °C is about 2 mM. The exact value was difficult to find. The determination of CMCs of bile salts is in general challenging owing to their stepwise aggregation [208] and a low aggregation number. Earlier, CMCs for taurocholic acid of 6 mM in 150 mM NaCl [18] and 10 mM in pure water have been suggested by Roda et al. [18]. In general, the CMC value of an ionic surfactant depends on the presence of electrolytes in the surfactant solution. All factors that lower the electrostatic repulsion between the charged hydrophilic head groups of ionic surfactants favor micelle formation [15]. Specifically, the many different aggregates of sodium taurocholate are stabilized by ion-ion and ion-dipole interactions, and by hydrogen bonds, and water and cations are essential in stabilizing the aggregates [211]. As compared with pure water, the repulsion between anionic taurocholate groups is noticeably attenuated in 15 mM ammonium acetate (pH 9.5, adjusted with 25% ammonia). Our low CMC value of about 2 mM is in agreement with the lowered repulsion.

In our PF-MEKC studies, the presence of sodium taurocholate, even at a concentration (1.5 mM) under the estimated CMC (2 mM), is crucial for the separation in the absence of ODMS. One explanation for this could be the competitive solubilization between taurocholate and the analytes into the SDS micelles following the non-specific mechanisms described by Wiedmer at al [15]. It is also possible that the migration of steroids is affected by the formation of complexes between steroids and taurocholate monomers via hydrogen bonds. In the sequential PF-MEKC mode, the taurocholate monomers, injected in the second pseudostationary phase, penetrate the first injected pseudostationary phase containing SDS and ODMS. Thus, the steroid analytes are partially separated before they meet the second resolving component, multiply charged ODMS. Steroid concentration occurs at this state, possibly due to sweeping by ODMS. Concentration was observed as a notable increase in the peak heights, especially for T, as compared with those in non-sequential modes.

The resolving power of ODMS, a derivatized γ–cyclodextrin, relies on its formation of inclusion complexes with the steroids [212]. The interactions between steroids and cyclodextrins are non-specific and relatively weak: association constants for steroids and γ–cyclodextrins between 2300 and 10000 M^{-1} have been reported [213, 214]. The formation of inclusion complexes is affected by temperature, steroid structure, and the matrix [213]. The presence of organic solvents, for

instance, results in lowered association constants [212, 213]. Accordingly, we chose not to use organic modifiers in our sequential PF-MEKC studies.

In study III, the sequential PF-MEKC method developed in study II, was employed for the determination of testosterone in male urine samples. Slight modifications to the method were made to improve the sensitivity and repeatability. Sensitivity was enhanced by utilizing the micellar sweeping effect. A search for the largest sample injection volume yielding linear correlations between steroid concentrations and peak areas revealed a value of 80 nL (10-fold the sample volume applied in study II). Sensitivity enhancement factors in terms of peak heights (SEF_{height}) between 4.6 (for T) and 9.8 (for fluoxymesterone) were obtained, and the LOD values were significantly lowered as a result (Table 10). However, as compared with the SEF_{height} values reported for T in other conditions (1500 [24] and 330 [32]), the effect of micellar sweeping was poor. The reduced performance of sweeping in our study (III) is due to the high pH resulting in fast EOF, the presence of an organic solvent (EtOH) in the sample matrix (to ensure solubility of T), and the partial filling approach suffering from gradual decrease of micelle quantity. Repeatability of the method was improved by equilibrating the capillary with the pseudostationary phase solution (SDS / sodium taurocholate) followed by BGE between runs. The PF-MEKC–UV results obtained in study III are compiled in Table 10, and the separation of F, A, T, MT, and E is presented in Figure 11.

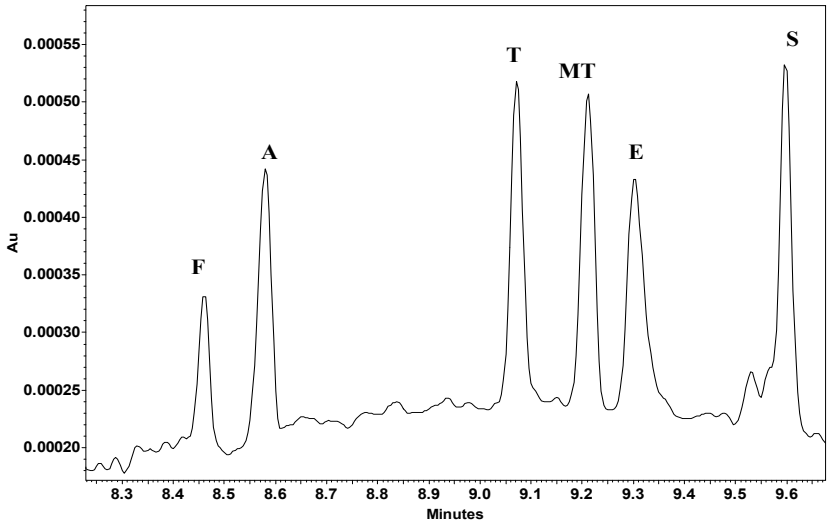

Figure 11. Separation of F, A, T, MT, and E (0.25 mg/L per each) by the sequential PF-MEKC method with use of sweeping (study III). Sample volume 80 nL. S is a system peak. Detection at 247 nM. Detailed analysis conditions in Table 6 (page 64).

Steroid identifications by ESI-MS and UV after similar PF-MEKC separations were investigated in study IV. Since the PF-MEKC method developed in studies II and III proved to be suitable for UV detection only (see section 5.3), a new PF-MEKC method without cyclodextrins, was developed. The method is similar to the one developed in study I (see Table 8), the most significant differences being the separation voltages (+ 25 kV (I) and +30 kV plus a back pressure of 0.1 psi (IV)), the concentration of sodium taurocholate (1.1 mM (I) and 1.5 mM (IV)), and the pseudostationary plug lengths (3.5 cm (I) and 2.8 (IV)). The PF-MEKC–UV results of study IV are listed in Table 10. Since this PF-MEKC method was optimized for ESI-MS coupling in the first place, the discussion is continued in section 5.3.

Relative to the few other quantitative capillary electromigration methods for T and related steroids (listed in Table 2, section 2.6), our optimized PF-MEKC–UV methods, especially the one developed in study III, show enhanced sensitivity, good repeatability, and excellent linearity. This is the first study in which a discontinous pseudostationary phase comprising two different solutions has been utilized. Interestingly, most of the analytes, including fluoxymesterone,

metandienone, 17-epimetandienone, and epitestosterone, have not been analyzed by any capillary electromigration techniques previously.

5.2 Direct infusion ESI-MS of steroids

Ionization of T and related steroids by ESI-MS in positive ion mode was investigated in studies I and IV. The data for F, A, T, MT, and E collected from the spectra are compiled in Table 11. Data for the ionization of metandienone and 17-epimetandienone are presented in publication I.

Table 11. ESI-MS behavior of testosterone and related steroids in positive ion mode.

Study		Analyte				
		F	**A**	**T**	**MT (i.s.)**	**E**
I, IV	Most intense ion (100%) (m/z, amu)	$[M+H]^+$ at 337 (I)	$[M+H]^+$ at 287	$[M+H]^+$ at 289	$[M+H]^+$ at 303	$[M+H]^+$ at 289 (IV)
I	Second most intense ion (m/z, amu)	$[M+Na]^+$ at 359 (6.3%)	$[M+Na]^+$ at 359 (17.5%)	$[M+Na]^+$ at 359 (19.0%)	$[M+Na]^+$ at 359 (17.7%)	-
IV	Second most intense ion (m/z, amu)	-	$[2M+H]^+$ at 574 (5%)	$[2M+H]^+$ at 578 (7.%)	$[2M+H]^+$ at 606 (9%)	$[2M+H]^+$ at 578 (9%)
I	Most intense daughter ions in the MS/MS mode (m/z, amu)	None	97, 109	97, 109	97, 109	-
IV	Most intense daughter ions in the MS/MS mode (m/z, amu)	-	97 (100%) 109 (75%)	97 (100%) 109 (90%)	97 (98%) 109 (100%)	97 (100%) 109 (95%)

All the investigated steroids except 17-epimetandienone produced their most intense ions at m/z corresponding to $[M + H]^+$. 17-Epimetandienone gave its strongest signal at m/z corresponding to $[M - H_20 + H]^+$. Protonated molecular ions are typically produced in ESI-MS, and also for steroids, as reported for T,

MT, E, and metandienone [205, 206]. In study I, the second most intense ions were sodium adducts, which are also often observed for steroids [205]. Interestingly, sodium adducts were not detected in study II. Instead, all steroids produced dimeric ions $[2M + H]^+$. The explanation for these differences might be the greater amount of steroids injected in study IV than in study I (see Table 7) resulting in suppressed ionization. Ammonium adducts, earlier reported for F [206], were not detected in either study, despite the presence of ammonium ions in the sample matrices.

In MS/MS mode, A, T, MT, and E produced fragments at m/z 97 and 109 amu. Such fragments are often detected in ESI-MS analyses of testosterone and related compounds having the 4-ene-3-one structure [206, 215, 216]. The ion at m/z 109 amu is formed by so-called B ring fission producing a residue of C_7H_9O [216], while the ion at m/z 97 amu represents a residue of C_6H_9O [216]. Some differences were observed in the relative abundances of these fragments, but they were insufficient for steroid differentiation. Importantly, the ESI-MS and ESI-MS/MS behaviors of testosterone and epitestosterone proved to be nearly identical. Fluoxymesterone did not produce any fragment ions, probably because the electronegative fluorine atom protected it from fragmentation.

5.3 PF-MEKC–ESI-MS of steroids

The analysis of steroids by PF-MEKC–ESI-MS was investigated in studies I and IV.

In study I, preliminary PF-MEKC–ESI-MS studies for F, A, and T were performed using the PF-MEKC method optimized for UV detection (described in publication I and Table 6). Only a few modifications to the method were made, namely, the separation was performed at +20 kV and 3447 Pa (0.5 psi) back pressure. Thermosetting the separation capillary was not possible.

The ESI-MS detection proved to be highly selective. Only the desired ions were detected on the selected channels ($[M + H]^+$). Separation of the analytes was successful even when the separation capillary was coupled to the ESI interface.

In study IV, PF-MEKC–ESI-MS of endogenous steroids A, T, and E was investigated with use of MT as internal standard. The hyphenation of PF-MEKC and ESI-MS was optimized by first searching for the best ESI-MS conditions and then optimizing the PF-MEKC separation. To optimize the sensitivity of the ESI-MS detection, ionization of testosterone (1 µg/mL) was tested in various organic solvents (Figure 12). The most intense signal of $[M + H]^+$ was detected in MeOH / 20 mM ammonium acetate (pH 9.5) (75/25, v/v) containing 0.1% of acetic acid. Almost as intense signals for testosterone were detected in EtOH / 20 mM ammonium acetate (pH 9.5) (75/25, v/v) and 2-propanol / 20 mM ammonium acetate (pH 9.5) (75/25, v/v), both containing 0.1% of acetic acid. In contrast to this, a relatively weak signal of $[M + H]^+$ was detected in the ACN-based solution. Because methanol has a higher relative permittivity value [217], lower autoprotolysis constant (pK_{auto}) [217], and lower evaporation temperature than ethanol and 2-propanol, we chose MeOH as sheath liquid solvent for the PF-MEKC–ESI-MS studies.

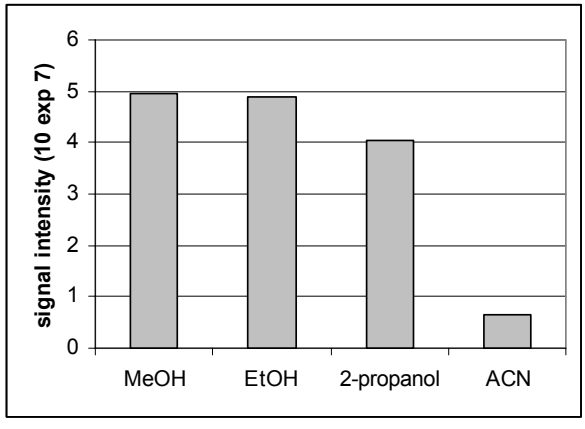

Figure 12. Signal intensity of the protonated molecular ion of testosterone in different solutions containing organic solvent / 20 mM ammonium acetate (pH9.5) / acetic acid (75/25/0.1%, v/v/v) in ESI-MS (positive ion mode).

Our starting point in study IV was the sequential PF-MEKC developed in study III. Unexpectedly, the method was not suitable for coupling with ESI-MS. The use of two different micellar solutions produced significant noise even when selected ion monitoring (SIM) was applied. The reason for this was not clear since in PF-MEKC the micelles and sulfated cyclodextrins migrate in the capillary slower than the electrically neutral analytes, as presented in publication II. One explanation could be the interference caused by the plentiful sodium ions

and some impurities (1% by weight sodium sulfate) in the cyclodextrin product. Importantly, the micellar sweeping effect could not be used as effectively in PF-MEKC–ESI-MS as in the PF-MEKC–UV method (discussed in section 5.1). In PF-MEKC–ESI-MS, the introduction of a sample volume of 80 nL (containing 3200 pg of four steroids in total) caused a labile electric field across the capillary. A loss of current was observed when the analytes exited the capillary.

In further studies, a new PF-MEKC–ESI-MS method, not relying on cyclodextrins, was developed and optimized. Total ion current (TIC) monitoring of the selected channels allowed good resolution for A, T, and E, but resolution between T, MT, and E was insufficient to allow their identification by TIC. However, all the steroids could be reliably identified through use of SIM analysis. The protonated molecular ions of T, MT, and E could be identified by SIM according to the differences in their migration times. Separation of T and E was crucial since their MS and MS/MS behaviors were identical (as discussed in section 5.2). The results were systematically compared with those obtained by corresponding PF-MEKC–UV (Table 12).

Table 12. Comparison of results for steroids (A, T, MT, and E) obtained by corresponding PF-MEKC–ESI-MS and PF-MEKC–UV methods.

Parameter	***Analyte***							
	A		***T***		***MT (i.s.)***		***E***	
	ESI-MS	***UV***	***ESI-MS***	***UV***	***ESI-MS***	***UV***	***ESI-MS***	***UV***
Migration time (min)	6.7	8.80	7.1	9.85	7.2	10.05	7.3	10.12
Peak width at 50% height (min)	0.15	0.05	0.11	0.05	0.15	0.04	0.13	0.05
LOD (μg/mL)	5	0.065	1.25	0.097	0.75	0.072	1.25	0.085
RSD% (peak area), absolute	74	2.2	62	1.4	52	2.6	61	1.4
RSD% (peak height), absolute	38	2.0	50	1.3	45	1.7	56	7.0
RSD% (migration time), absolute	1.9	0.6	3.3	0.5	3.8	0.5	4.1	0.5
RSD% (peak area), relative	42	1.7	22	3.1	-	-	20	2.8
RSD% (peak height), relative	30	1.8	23	1.1	-	-	13	6.1
RSD% (migration time), relative	0.7	0.09	0.6	0.09	-	-	0.5	0.04

In comparison with the corresponding PF-MEKC–UV method, the PF-MEKC–ESI-MS method suffered from reduced resolution, repeatability, and sensitivity. The elevated temperature of the separation capillary, which could not be thermostated in PF-MEKC–ESI-MS, was an important reason for the reduced resolution. Even though the use of an internal standard significantly enhanced the RSD values in PF-MEKC–ESI-MS, PF-MEKC–UV was the more suitable method for quantitative analysis of steroid standards. Furthermore, in-capillary sample concentration by micellar sweeping could be more efficiently utilized in PF-MEKC–UV than in PF-MEKC–ESI-MS. In PF-MEKC–ESI-MS, sweeping of steroids, more than 1600 pg in total, caused frequent difficulties in maintaining the electric field across the capillary. This is reasonable to expect since the electrically neutral steroid zones work as resisting medium in the capillary. Because the electric field in PF-MEKC–ESI-MS was already weakened by the low conductivity of the MeOH-based sheath liquid (in comparison with the aqueous electrolyte solution in PF-MEKC–UV), and the positive voltage applied in the ESI needle, the PF-MEKC–ESI-MS field was highly sensitive to any disturbing factors. Furthermore, the formation of dimeric ions at high steroid concentrations (detected for testosterone at 7.5 μg/mL) most likely decreased the current. We discovered that in PF-MEKC analyses of electrically neutral steroids, ESI-MS interfacing sets significant limitations not only on the chemistry affecting the ionization and detection processes but also on the PF-MEKC separation itself.

Our PF-MEKC–ESI-MS studies of T and related steroids are the first to consider the MS identification of steroids after separation by any capillary electromigration technique. Furthermore, limitations of sweeping in PF-MEKC–ESI-MS have not been discussed before.

5.4 Non-specific SPE of steroids in urine samples

Non-specific SPE of A, T, and E in spiked urine samples was performed in study II. The recoveries of the analytes were excellent: 105% (RSD 7.6%) for A, 92% (RSD 5.4%) for T, and 91% (RSD 7.6%) for E. After sequential PF-MEKC analysis of the spiked urine after non-specific SPE, the steroids were easily discriminated from the matrix. Analysis of steroid-free male urine revealed a non-disturbing matrix effect.

However, analysis of steroids (T, E) in natural male urine samples by sequential PF-MEKC–UV method (developed in study III) after enzymatic hydrolysis with *β*-glucuronidase and non-specific SPE was not successful (unpublished data). Significant interference from aromatic chromophores deriving from samples obscured the signals of steroids.

5.5 Analysis of the recombinant anti-testosterone Fab fragment and preparation of anti-T immunosorbent

In study III, an anti-T immunosorbent was prepared to develop a new specific IA-SPE method for testosterone in urine.

The recombinant anti-T Fab fragment (Fab220), kindly donated by Prof. Kristiina Takkinen, was analyzed by SDS-PAGE (Figure 13).

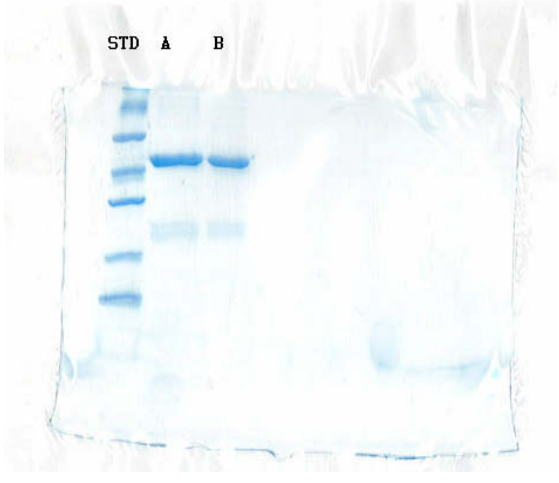

Figure 13. SDS-PAGE of recombinant anti-T Fab fragment (Fab220) stained with Coomassie Blue under nonreducing conditions. STD is a standard solution of molecular weight markers, A and B are identical samples of Fab220.

The SDS-PAGE showed that the fragment was approximately 95% pure and that an interchain disulfide bond was formed between the light and heavy chains. According to the results obtained by ELISA, the affinity between the anti-T Fab fragment and testosterone (actually testosterone-3-(O-carboxymethyl)oxime-

BSA) was very high (EC_{50}, effective concentration required to induce 50% response, 2.8 nM, unpublished data). Our result is in relatively good agreement with the EC_{50} value previously reported for Fab220 by Lu et al. [184] (2.8 ± 0.3 ng/mL, *i.e*. 9.7 ± 1.0 nM). In comparison with the affinity reported between another anti-T antibody, 3-C_4F_5, and T (30 nM) [183], the affinity between Fab220 and T is about ten times higher.

Immobilization of the Fab fragment on activated Sepharose was effective. Thanks to immobilization *via* the histidine tag, located at the heavy chain opposite to the testosterone binding site, the orientation of the Fab fragment was active. According to the absorbance measurements at 280 nM, 98.8–99.4% of the Fab fragment was coupled to Sepharose. Supporting results were obtained by the BCA Protein Assay and SDS-PAGE. The immobilization efficiency is in good agreement with that earlier reported by Nevanen et al. [95–98%, 187]. The Fab concentration of the immunosorbent was 25 mg/mL. Theoretical binding capacity of the immunosorbent (0.53 nMol/µL, i.e. 150 ng/ µL) was calculated by assuming that the binding stoichiometry of a Fab fragment and a testosterone molecule is 1:1. The free light chains of the Fab fragment (~ 5%) were eluted from the immunosorbent by preliminary elution with EtOH / 30 mM ammonium acetate (pH 7.4) (50/50, v/v) before extractions.

5.6 Immunoaffinity solid-phase extraction of testosterone and subsequent analysis by PF-MEKC–UV

The efficiency of the IA-SPE of testosterone was determined by loading testosterone standards corresponding to 90% of the theoretical binding capacity into a 5-µL immunosorbent column as described in section 4.3.9. After elution with EtOH / 30 mM ammonium acetate (pH 9.5) (50/50, v/v), T was determined by PF-MEKC–UV. The recovery of T was 90% (RSD 15.5%, n = 15), similar to the recovery obtained by non-specific SPE (92%, RSD 5.4%). After elution with EtOH / HEPES (pH 7.4) (50/50, v/v), the recovery was 92% (RSD 20.1%, n = 18). The IA-SPE recovery results indicate that at least 81% of the immobilized Fab fragments were active and able to capture one testosterone molecule each. The binding capacity was not decreased during repeated extraction-elution cycles performed in one day. The results are in good agreement with those of previous IA-SPE studies performed with another Fab fragment [187].

Steroid-free female urine spiked with T, corresponding to 50% of the theoretical binding capacity of the immunosorbent, was analyzed by IA-SPE and PF-MEKC–UV. Recovery of T was excellent: 91% (RSD 12.5%). A linear correlation between T concentrations (35–750 μg/L in steroid-free female urine) and peak areas was obtained with r^2 of 0.989. The LOQ value for T was 35 μg/L.

Finally, after enzymatic hydrolysis with *β*-glucuronidase from *E. coli*, three different male urine samples were analyzed for T. The samples were concentrated 3.4-fold during IA-SPE. Concentration of T in urine samples varied between 20 μg/L (RSD 20.5%) and 43 μg/L (RSD 12.1%). The results are in good agreement with the T concentrations in urine reported by other groups [118, 119].

The developed IA-SPE, based on the use of a recombinant Fab fragment optimally immobilized on a solid support, proved to be an efficient and fast sample pretreatment method for determination of T in male urine samples by PF-MEKC–UV. In comparison with non-specific SPE, discussed in section 5.4, IA-SPE provided a much more effective elimination of matrix components. The overall analysis time, including IA-SPE and PF-MEKC–UV, for a sample was just 14 min when the hydrolysis time (60 min) is not included. Importantly, the resolving power of PF-MEKC–UV was beneficial in view of the minor cross-reactivity (7.2%) reported between the anti-T Fab fragment and epitestosterone [184]. Although the PF-MEKC–UV method (LOD 18 μg/L for T) is not as sensitive as immunoassays, GC–MS or LC–MS, the obtained LOQ enables determination of T in small volumes (0.8–1.0 mL) of male urine after IA-SPE. Unfortunately, the RSD values of IA-SPEs were relatively high, perhaps due to the manual approach that was used (see section 4.3.9). Automated extraction-elution cycles could be expected to result in better repeatability.

Our IA-SPE application for testosterone is the first to be described. Owing to the small size of the Fab fragment (~ 50 kDa) and the efficient immobilization method, a high concentration of active Fab fragments in the immunosorbent was achieved. This is an important advantage since only 5 μL of IS was required to prepare an appropriate IA-SPE column. In comparison with ratios used in IACE applications (15:1 [93] and 50:1 [98]), a low ratio of 2:1 of the Fab fragment to T was adequate for the quantitative capture of T from spiked urine (45 μg/L). Despite the stabile binding capacity of IS during one day, we preferred the use of fresh (unused) IS for each male urine sample to avoid interefence between samples.

The determination of T in male urine samples by microscale IA-SPE and subsequent PF-MEKC–UV was clearly successful. Our method provides many attractive features. Compared with traditional methods for the determination of T in male urine it is specific (ensured by both the specific recombinant anti-T Fab fragment and efficient PF-MEKC), simple (no need for derivatization and multiple, often non-specific extraction steps) and fast. However, automation of the method will be required before it can be considered for routine use.

5.7 Hyphenated IA-SPE–PF-MEKC–UV analysis of testosterone

In study III, our original motivation was to perform in-capillary IA-SPE and subsequent analysis of T by PF-MEKC–UV. The idea was to concentrate T inside the capillary prior to analysis with a view to improving the sensitivity. Another aim was to develop an efficient method without manual steps. The hyphenated application is related to IACE. Previously, IACE, based on immobilized antibody (fragments), has only been performed for charged ligands [83, 84, 87–89]. We expected that thorough investigation of the chemistry and methodology, together with proper timing of the sample introduction, extraction, rinsing, elution and electromigration steps, would result in an efficient in-capillary analysis method for a neutral ligand, too.

Unfortunately, despite the careful optimization of the hyphenated IA-SPE–PF-MEKC–UV, the method could not be stabilized, and the results were at best only indicative of the presence of T (standard). The challenge of the method is the compulsory use of surfactants, which have a destructive effect on the protein structures. Thus, contact between the pseudostationary phase and the immunosorbent has to be avoided if the method is to be repeatable. The pseudostationary phase had to be introduced to the capillary from the detector side, after introduction of the eluant plug. This resulted in great challenges to the controlled (repeatable) transport of solutions inside the capillary. Most likely, the construction of the hyphenated IA-SPE–PF-MEKC–UV system was not robust enough to meet these challenges. Further, the sequential PF-MEKC–UV method, developed in study II, could not be employed in the hyphenated IA-SPE–PF-MEKC–UV owing to interferences by the steroid backbone of taurocholate at the steroid identification wavelength (247 nM). Consequently, in the hyphenated application,

the cross-reactivity between the Fab fragment and E [184] could not be effectively compensated.

5.8 Determination of association constants between steroids and both human and bovine serum albumins

In study V, an algorithm presented by Nilsson et al. [76] was modified and extended so as to be valid for neutral ligands, and to compensate variations in the EOF. Calculations of the dissociation constants, K_d, for steroids and fatty acid free-HSA were performed using *Eq.* 10 (section 4.3.11). Linear correlations were obtained between the molar amount of fatty acid-free HSA (n) and change in the steroid migration times (Δt), with r^2 0.997 or higher for all steroids. The affinity data between the steroids and fatty acid-free HSA is compiled in Table 13. The association constants, K_b, between HSA and testosterone, epitestosterone, androstenedione, methyltestosterone, and fluoxymesterone were 32100 ± 3600 M^{-1}, 21600 ± 1500 M^{-1}, 13300 ± 1300 M^{-1}, 34100 ± 200 M^{-1}, and 8500 ± 300 M^{-1}, respectively. The K_b values found for testosterone and fatty acid-free HSA are in good agreement with the values found by other techniques (K_b 28000 ± 2000 M^{-1}, log K_b 4.43 ± 0.02 at 20 ºC, obtained by equilibrium dialysis [112] and K_b 33000 ± 3000 M^{-1}, log K_b 4.52 ± 0.04 at 25 ºC [114], obtained by equilibration reaction followed by the determination of unbound steroid by HPLC). The interactions between epitestosterone and HSA were substantially weaker than those between testosterone and HSA. This is interesting because T and E differ only in the spatial orientation of the hydroxyl group. Even weaker interactions were observed between androstenedione and HSA and fluoxymesterone and HSA. In contrast to this, the interactions between methyltestosterone and HSA were slightly stronger than those between T and HSA.

We also measured the association constants between the steroids and impure HSA (not effectively purified from plasma fatty acids). Fatty acids have strong interactions with albumins (K_b values at 5 °C for example 6.7×10^7 M^{-1} for laurate and HSA, and 2.3×10^8 M^{-1} for myristate and HSA [218]), and the availability of steroids to tissues may be affected by binding of fatty acids to albumin [114]. Our interest was to study whether possible fatty acid residues present in crystallized HSA affect the binding of steroids to HSA after

solubilization. The affinity data between steroids and impure HSA is compiled in Table 13. No differences in binding of steroids to fatty acid free HSA and impure HSA were observed in the experiments,. Similar results have been reported by Watanabe and Sato [114], who observed no change in the K_b values for testosterone and HSA when the molar ratio of palmitic acid to HSA was varied between 0 and 1:1.

Table 13. Apparent association constants, K_b, for the binding of testosterone, epitestosterone, androstenedione, methyltestosterone, and fluoxymesterone to fatty acid-free HSA and impure HSA (without fatty acids removed) at +37 °C.

	HSA, fatty acid-free		**HSA, impure**	
	K_b (M^{-1})	log K_b	K_b (M^{-1})	log K_b
Testosterone	32100 ± 3600	4.50 ± 0.05	27900 ± 2100	4.45 ± 0.03
Epitestosterone	21600 ± 1500	4.34 ± 0.04	21000 ± 700	4.32 ± 0.01
Androstenedione	13300 ± 1300	4.12 ± 0.04	11700 ± 500	4.07 ± 0.02
Methyltestosterone	34100 ± 200	4.53	32900 ± 2600	4.52 ± 0.04
Fluoxymesterone	8500 ± 300	3.93	9100 ± 1700	3.95 ± 0.13

Similar measurements were performed for steroids and BSA (both fatty acid-free and impure). Linear correlations between the molar amount of fatty acid-free BSA and the change in the steroid migration times were excellent (r^2 0.997 or better for all steroids). The affinity data for binding between the steroids and BSAs is listed in Table 14. All in all, the relative affinities between the steroids and BSAs were similar to those between steroids and HSA. No clear differences in interactions between steroids and impure BSA and between steroids and fatty acid-free BSA were observed. Interestingly, interactions between steroids and BSA were about 1.5-fold weaker than those between steroids and HSA. An explanation for this may be the differences in the albumin structures, assuming that the steroid binding sites are the same in the two proteins.

Table 14. Apparent association constants, K_b*, for the binding of testosterone, epitestosterone, androstenedione, methyltestosterone, and fluoxymesterone to fatty acid-free BSA and impure BSA (without fatty acids removed) at +37 °C.*

	BSA, fatty acid-free		**BSA, impure**	
	K_b (M^{-1})	log K_b	K_b (M^{-1})	log K_b
Testosterone	18800 ± 1500	4.27 ± 0.04	20600 ± 2200	4.31 ± 0.04
Epitestosterone	14000 ± 400	4.15 ± 0.01	14100 ± 600	4.15 ± 0.01
Androstenedione	7800 ± 900	3.89 ± 0.06	7200 ± 400	3.86 ± 0.03
Methyltestosterone	21100 ± 3000	4.32 ± 0.06	21700 ± 800	4.34 ± 0.01
Fluoxymesterone	6400 ± 400	3.81 ± 0.03	8000 ± 1000	3.91 ± 0.05

Association constants for the binding of T-related steroids with albumins were little studied before this work. Except for values reported for T and HSA, and T and BSA [112–114], association constants for binding of T-related steroids (androstenedione, epitestosterone, fluoxymesterone, and methyltestosterone) with albumins did not exist. The new data obtained here may be of considerable importance for endocrinologists. Particularly interesting may be the differences in affinities between testosterone and HSA and epitestosterone and HSA, since epitestosterone is considered to have antiandrogenic activity [121, 122]. The biological activity of a steroid (*i.e.* its access to receptors) is affected by interactions with HSA [219], and hence the weaker interactions between epitestosterone and HSA than between testosterone and HSA may have a physiological significance.

The new algorithm developed in study V provides an easy way to determine the association constants for proteins and neutral ligands. It also allows the variations in EOF to be taken into account. Before this work, all ACE studies focusing on neutral ligands were based on a competitive approach [57–59]. However, the competitive approach suffers from the common disadvantages associated with derivatization [82]. Furthermore, it may also suffer from corrupted data owing to differences in the affinities between the protein and small, actual (non-labeled) and labeled ligands. The new algorithm and the PF-ACE method that were developed provide a straightforward and easy approach

for affinity determinations. In comparison with techniques, such as SPR and HPLC, PF-ACE offers significant benefits for affinity measurements, including easily controlled temperature and protein and ligand concentrations, and performance in free solution (without need for unnatural immobilization procedures), relative to the situation in plasma.

6. Conclusions

New efficient methods were developed for the determination of testosterone and related steroids. With optimized pseudostationary phases comprising surfactants and cyclodextrins, partial filling micellar electrokinetic chromatography provided an effective technique for the separation of these structurally similar compounds. A discontinuous pseudostationary phase, consisting of two different solutions, provided greater separation efficiency than the corresponding system where solutions were employed separately. A moderate improvement of the sensitivity in UV detection was obtained by micellar sweeping.

Comparison of ESI-MS and UV detections after similar PF-MEKC separations showed that, in the analysis of electrically neutral steroids, UV allows higher sensitivity, repeatability and peak resolution. The method of analyte concentration by micellar sweeping could be more efficiently utilized in UV than in ESI-MS. Furthermore, interfacing with ESI-MS resulted in significant limitations not only in the chemistry affecting the ionization and detection processes, but in the separation itself.

PF-MEKC–UV was tested in the determination of testosterone in male urine samples. Enzymatic hydrolysis by *β*-glucuronidase from *E. coli* was employed in the deconjugation of steroid glucuronides. Non-specific SPE, performed with a surface-modified styrene divinylbenzene polymer, was not a suitable sample pretreatment method before PF-MEKC–UV analysis owing to the significant interference from the matrix. However; a new immunoaffinity solid-phase extraction method, relying on specific interactions between testosterone and a recombinant anti-testosterone antibody Fab fragment, proved to be an excellent off-line pretreatment method for male urine samples before PF-MEKC–UV analysis. After the hydrolysis and IA-SPE procedures, no further sample treatment was required. Previous examples of IA-SPE of testosterone do not exist, probably due to difficulty of producing effective anti-testosterone antibodies by the hybridoma technique. Here, an effective and highly specific Fab fragment, produced by the phage display technique, was efficiently utilized after immobilization in a highly oriented manner. In its specificity, simplicity, and rapidity, the new method (IA-SPE and subsequent analysis by PF-MEKC–UV) for determination of testosterone in male urine provides an attractive alternative to traditional approaches.

Despite the careful optimization of the hyphenated IA-SPE–PF-MEKC–UV, the method could not be stabilized, and the results were only indicative of the presence of T (standard).

Finally, new data describing the interactions of testosterone-related steroids with albumin proteins was obtained by PF-ACE. Notable differences in the association constants were found. Especially interesting were the affinity differences between testosterone and HSA and epitestosterone and HSA. The biological activity of a steroid is affected by its interactions with HSA, and hence the weaker interactions between epitestosterone and HSA than between testosterone and HSA may be of physiological significance. The new algorithm introduced in this study offers a straightforward method to determine the association constants between proteins and neutral ligands. In comparison with other techniques for affinity measurements, PF-ACE is more accurate and provides results that reflect the equilibrium as it exists in solution.

In sum, the new capillary electromigration methods provide versatile tools for steroid analysis. The use of non-specific interactions between analytes and electrolyte additives makes possible an efficient separation and quantification of steroids. Finally, the use of specific interactions between steroids and biomolecules may provide interesting and accurate data difficult to obtain by any other technique.

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Use of non-specific and specific interactions in the analysis of testosterone and related compounds by capillary electromigration techniques

Abstract

Determination of testosterone and related compounds in body fluids is of utmost importance in doping control and the diagnosis of many diseases. Capillary electromigration techniques are a relatively new approach for steroid research. Owing to their electrical neutrality, however, separation of steroids by capillary electromigration techniques requires the use of charged electrolyte additives that interact with the steroids either specifically or non-specifically. The analysis of testosterone and related steroids by non-specific micellar electrokinetic chromatography (MEKC) was investigated in this study. The partial filling (PF) technique was employed, being suitable for detection by both ultraviolet spectrophotometry (UV) and electrospray ionization mass spectrometry (ESI-MS). Efficient, quantitative PF-MEKC–UV methods for steroid standards were developed through the use of optimized pseudostationary phases comprising surfactants and cyclodextrins. PF-MEKC–UV proved to be a more sensitive, efficient and repeatable method for the steroids than PF-MEKC–ESI-MS. We discovered that in PF-MEKC analyses of electrically neutral steroids, ESI-MS interfacing sets significant limitations not only on the chemistry affecting the ionization and detection processes, but also on the separation. The new PF-MEKC–UV method was successfully employed in the determination of testosterone in male urine samples after microscale immunoaffinity solid-phase extraction (IA-SPE). The IA-SPE method, relying on specific interactions between testosterone and a recombinant anti-testosterone Fab fragment, is the first such method described for testosterone. Finally, new data for interactions between steroids and human and bovine serum albumins were obtained through the use of affinity capillary electrophoresis. A new algorithm for the calculation of association constants between proteins and neutral ligands is introduced.

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Nimeke

Epäspesifisten ja spesifisten vuorovaikutuksien hyödyntäminen testosteronin ja sen sukuisten steroidien kapillaarielektromigraatioanalyyseissä

Tiivistelmä

Testosteronin ja sen sukuisten steroidien määrittäminen kehon nesteistä on keskeistä doping-valvonnassa sekä monien sairauksien diagnosoinnissa. Kapillaarielektromigraatiotekniikat ovat varsin uusi lähtökohta steroiditutkimuksille. Steroidien sähköisestä varauksettomuudesta johtuen kapillaarielektromigraatiotekniikoiden soveltaminen steroidien erottamiseen edellyttää ionisoituvien orgaanisten lisäaineiden käyttöä elektrolyyttiliuoksessa. Lisäaineilla voi olla joko spesifisiä tai epäspesifisia vuorovaikutuksia steroidien kanssa. Tässä tutkimuksessa testosteronin ja sen sukuisten steroidien määrittämistä tutkittiin epäspesifisellä misellisellä sähkökineettisellä kromatografialla (MEKC). Osittaistäyttötekniikan (PF) ansiosta steroidit voitiin tunnistaa sekä ultraviolettispektrofotometrisesti (UV) että sähkösumutus-ionisaatiomassaspektrofotometrisesti (ESI-MS). Steroidistandardeille kehitettiin tehokkaat ja kvantitatiiviset PF-MEKC–UV-menetelmät käyttäen lisäaineina tensidejä sekä syklodekstriinejä. PF-MEKC–UV osoittautui herkemmäksi, tehokkaammaksi ja toistettavammaksi menetelmäksi kuin PF-MEKC–ESI-MS. Tutkimuksessa havaittiin, että ESI-MS-liitäntä asettaa huomattavia kemiallisia rajoituksia paitsi ionisaatio- ja detektointitapahtumille, myös erotustapahtumalle. Uutta PF-MEKC–UV-menetelmää voitiin käyttää testosteronin määrittämiseen miesten virtsanäytteistä miniatyrisoidun immunoaffiniteettikiinteäfaasiuuton (IA-SPE) jälkeen. Kyseinen IA-SPE perustuu vuorovaikutuksiin testosteronin ja sille spesifisen rekombinantin Fab-fragmentin välillä. Aikaisemmin IA-SPE:tä ei ole sovellettu testosteronin uuttamiseen. Lopuksi, uutta tietoa steroidien sekä ihmisen ja naudan seerumin albumiinin välisistä vuorovaikutuksista saatiin affiniteettikapillaarielektroforeesin avulla. Tutkimuksessa kehitettiin uusi algoritmi, jonka avulla voidaan laskea proteiinien ja neutraalien ligandien välisiä sitoutumisvakioita.

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