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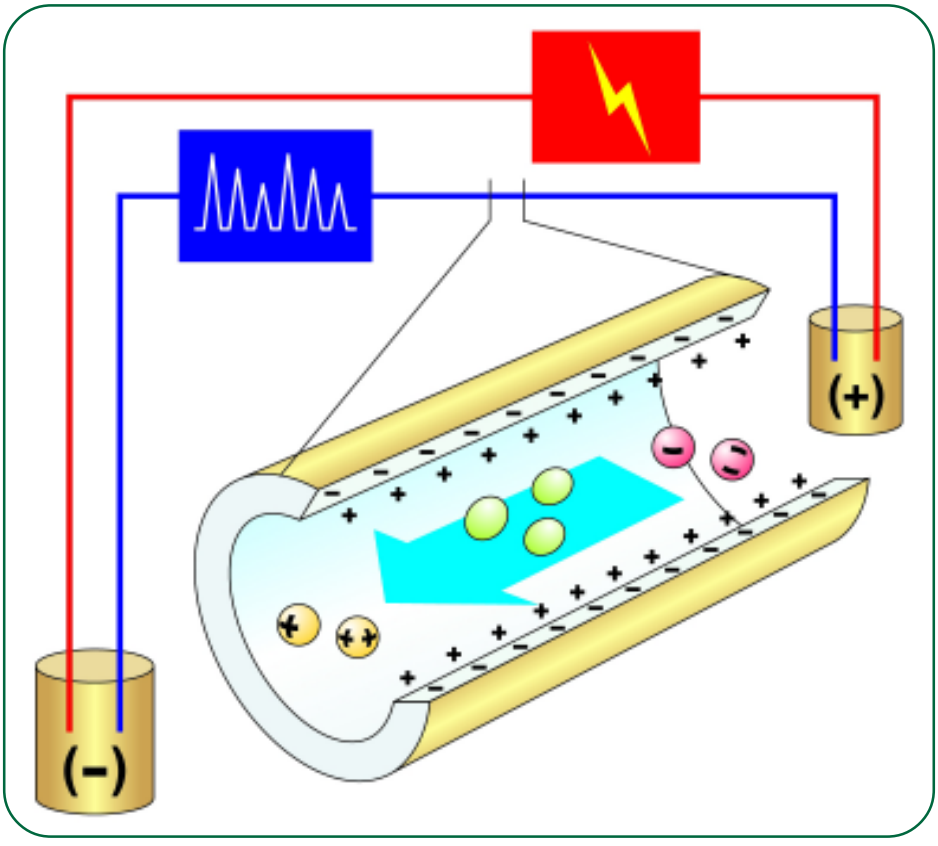

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Use of non-specific and specific interactions in the analysis of testosterone and related compounds by capillary electromigration techniques

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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.

To all my wonderful friends and family, a great big thanks for your support and interest in my work, and for the many wonderful days we have spent together. Specifically; Hanna, Lasse, Mikko, Eve, Juha, Olli, Minna, Maria, Kalle, Mia, and Sigbjørn, you mean so much to me. Kiitos Mummi! Tack Hammu! Kiitos Heimo! Tusen takk til familie og venner i Norge. Kiitos Annika! Hanna Katrine and Katri are thanked for helping our family while we lived in Munich.

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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.