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LINDA AHONEN Method Development for the Analysis of Steroids, Vitamin D and Oxysterols 24/2014

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LINDA AHONEN

Method Development for the Analysis of Steroids, Vitamin D and Oxysterols

DIVISION OF PHARMACEUTICAL CHEMISTRY AND TECHNOLOGY
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI

Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

METHOD DEVELOPMENT FOR THE ANALYSIS OF STEROIDS, VITAMIN D AND OXYSTEROLS

Linda Ahonen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2, Korona Information Centre, on October 3rd, at 12 noon.

Helsinki 2014

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ABSTRACT

Steroids, vitamin D and oxysterols are all part of a bigger group of compounds called lipids, which are generally important in the function of the human body. Steroids, vitamin D and oxysterols are present throughout the human body in a broad range of concentrations. In many cases the monitoring of these analytes can be of high value. Therefore, the development of a sensitive and universal method, suitable for the simultaneous analysis of these compounds, is of a great importance. Methods developed for the analysis of these compounds have to be highly selective and sensitive, which is a remarkable analytical challenge. The tremendous number of compounds and the complexity of different biological matrixes are also challenging for analysts.

In this study, the goal was to develop and test new mass spectrometry based methods for analyzing steroids, vitamin D and oxysterols. First, the feasibility of capillary liquid chromatography-microchip atmospheric pressure photoionization-tandem mass spectrometry (capLC-μAPPI-MS/MS) was examined for the analysis of anabolic steroids in human urine. The urine samples were enzymatically hydrolysed and the compounds were liquid-liquid extracted with diethyl ether. The capLC-μAPPI-MS/MS method showed good sensitivity, linearity and repeatability and the results demonstrate that the microchip APPI combined with capLC-MS/MS potentially provides a new method for analyzing non-polar and neutral compounds in biological samples.

Second, a microfluidic-based liquid chromatography-electrospray ionization-mass spectrometric system (HPLC-Chip/ESI/MS) was studied and compared to a conventional narrow-bore liquid chromatography-electrospray/mass spectrometric system (LC-ESI/MS) for the analysis of steroids. The limits of detection for oxime derivatized steroids, expressed as concentrations, were slightly higher with the HPLC-Chip/MS system, which used an injection volume of 0.5 μL than with the conventional LC-ESI/MS, which used an injection volume of 40 μL. When the limits of detection are expressed as injected amounts, however, the sensitivity of the HPLC-Chip/MS system was about 50 times higher compared to the conventional LC-MS system. The results clearly indicate that the use of the HPLC-Chip/MS system for the analyses of low-volume samples is advantageous. Additionally, the feasibilities of the HPLC-Chip/MS system in the analyses of non-derivatized and oxime derivatized steroids were compared and the HPLC-Chip/MS method developed for the non-derivatized steroids was successfully used in the quantitative analysis of 15 mouse plasma samples.

An ion mobility mass spectrometry method using a compact traveling wave cell (TWIM-MS) was developed for the separation of steroid isomers in addition to the miniaturized analytical methods. Three steroid isomer pairs

were analyzed in their native form and as their *p*-toluenesulfonyl isocyanate derivatives. The native steroids were separated from each other, but no separation could be attained for the isomers. The derivatized steroid isomers were, however, properly separated by TWIM-MS with peak-to-peak resolutions close to or as high as baseline resolution. In conclusion, the developed TWIM-MS method provides a reliable, fast and repeatable method for separating derivatized steroid isomers.

Finally, an ultra-high-performance liquid chromatography-atmospheric pressure photoionization-tandem mass spectrometric (UHPLC-APPI-MS/MS) method was developed for the simultaneous quantitative analysis of several oxysterols and vitamin D related compounds in mouse brain and cell line samples. An UHPLC-APPI-high resolution mass spectrometric (UHPLC-APPI-HRMS) method was developed for confirmatory analysis and for the identification of non-targeted oxysterols. Both developed methods showed good quantitative performance and APPI also provides high ionization efficiency for oxysterols and vitamin D related compounds without the time consuming derivatization step needed in the commonly used ESI. Several oxysterols were quantified in the mouse brain and cell line samples. Additionally, 25-hydroxyvitamin D_3 was detected in mouse brain samples for the first time.

In conclusion, the methods developed throughout this work are highly sensitive and selective new methods for the analysis of the selected lipids from biological samples. These methods provide new insights in the analysis of steroids, vitamin D related compounds and oxysterols, and with some additional development some of these presented methods could be implemented in routine laboratories such as those that conduct forensic or doping analysis.

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Helsinki, August 2014

Linda Ahonen

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Linda Ahonen, Markus Haapala, Ville Saarela, Sami Franssila, Tapio Kotiaho, and Risto Kostiainen. Feasibility of capillary liquid chromatography/microchip atmospheric pressure photoionization mass spectrometry in analyzing anabolic steroids in urine samples. *Rapid Communications in Mass Spectrometry* 2010, 24, 958-964.

II Linda Ahonen, Pekka Keski-Rahkonen, Taija Saarelainen, Jenni Paviala, Raimo A. Ketola, Seppo Auriola, Matti Poutanen, and Risto Kostiainen. Comparison of liquid chromatography-microchip/mass spectrometry to conventional liquid chromatography-mass spectrometry for the analysis of steroids. *Analytica Chimica Acta* 2012, 721, 115-121.

III Linda Ahonen, Maíra Fasciotti, Gustav Boije af Gennäs, Tapio Kotiaho, Romeu Daroda, Marcos Eberlin, and Risto Kostiainen. Separation of steroid isomers by ion mobility mass spectrometry. *Journal of Chromatography A* 2013, 1310, 133-137.

IV Linda Ahonen, Florian B.R. Maire, Mari Savolainen, Jaakko Kopra, Rob J. Vreeken, Thomas Hankemeier, Timo Myöhänen, Petri Kylli, and Risto Kostiainen. Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure photoionization-mass spectrometry. *Journal of Chromatography A* DOI: 10.1016/j.chroma.2014.08.088.

These publications are hereafter referred to in the text by their roman numerals.

Author's contribution to the publications included in this thesis:

I The experimental work, excluding the microfabrication, was carried out by the author. The manuscript was written by the author with contributions from the co-authors.

II All microchip/mass spectrometry based experimental work (including sample pretreatment) was carried out by the author together with Taija Saarelainen or with Pekka Keski-Rahkonen. The conventional liquid chromatography-mass spectrometry work (including sample pretreatment) was carried out by Pekka Keski-Rahkonen, and the mouse plasma samples were obtained by Jenni Paviala. The manuscript was written by the author with contributions from the co-authors.

III The mass spectrometry data were acquired by the author with contributions from Maíra Fasciotti and the nuclear magnetic resonance related work was done by Gustav Boije af Gennäs. The manuscript was written by the author with contributions from the co-authors.

IV The experimental work was carried out by the author (the triple quadrupole experiments) and by Petri Kylli (the quadrupole-time of flight experiments) with some assistance from Florian B.R. Maire. The mice brain samples were obtained by Jaakko Kopra and the cell line samples by Mari Savolainen. The manuscript was written by the author with contributions from co-authors.

GENERAL ABBREVIATIONS

A_s	peak asymmetry factor
AAS	anabolic androgenic steroids
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
CapLC	capillary liquid chromatography
CE	collision energy
CYP	cytochrome P450
dc	direct current
ECD	electron capture detector
EI	electron ionization
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
eV	electron volt
FID	flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GP	Girard P hydrazine
HMP	2-hydrazino-1-methylpyridine
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
i.d.	internal diameter
IMS	ion mobility spectrometry
IMS-MS	ion mobility spectrometry mass spectrometry
ISTD	internal standard
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LLOQ	lower limit of quantitation
LOQ	limit of quantitation
m/z	mass-to-charge ratio
MS	mass spectrometry/mass spectrometer
MS/MS	tandem mass spectrometry
NFPH	2-nitro-4-trifluoromethylphenylhydrazine
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
R^2	coefficient of determination
$R_{p\text{-}p}$	peak-to-peak resolution
RIA	radioimmunoassay
RP-LC	reversed phase liquid chromatography

rpm	rounds per minute
%RSD	relative standard deviation
S/N	signal-to-noise ratio
SPE	solid-phase extraction
SRM	selected reaction monitoring
TMS	trimethylsilyl
t_R	retention time
TWIM	traveling wave ion mobility
TWIM-MS	traveling wave ion mobility mass spectrometry
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet
W_b	peak width
W_h	peak half-width
WT	wild type
µAPPI	microchip atmospheric pressure photoionization

ABBREVIATIONS OF ANALYTES

A	aldosterone
AN	androstenedione
AND or 3α-AN	androsterone
D_3	cholecalciferol
d_3-D_3	d_3-cholecalciferol
CORT	corticosterone
CS	cortisone
7-DHYD	7-dehydrocholesterol
DHEA	dehydroepiandrosterone
11-DC	11-deoxycortisol
DESMO	desmosterol
DHT	dihydrotestosterone
1α,25-D_3	1α,25-dihydroxycholecalciferol
17-MDN	17-epimetandienone
17α-T	epitestosterone
D_2	ergocalciferol
α-ES	α-estradiol
β-ES	β-estradiol
ES	estrone
HC	hydrocortisone
25-OH-D_3	25-hydroxycholecalciferol
7α-OH-Chl	7α-hydroxycholesterol
7β-OH-Chl	7β-hydroxycholesterol
22-OH-Chl	22(*S*)-hydroxycholesterol
24-OH-Chl	24(*S*)-hydroxycholesterol
27-OH-Chl	27-hydroxycholesterol
d_6-27-OH-Chl	d_6-27-hydroxycholesterol
17-OH-PREG	17-hydroxypregnenolone
17-OH-PROG	17-hydroxyprogesterone
7-OXO	7-ketocholesterol
NANm	metabolite of nandrolone
MTS	methyltestosterone
NAN	nandrolone
PREG	pregnenolone
PROG	progesterone
T or 17β-T	testosterone
d_3-T	d_3-testosterone
3β-AN	trans-androsterone

1 REVIEW OF THE LITERATURE

Steroids, vitamin D and oxysterols are all part of a larger group of compounds called lipids. Generally, lipids are defined as naturally occurring small compounds, which are readily soluble in organic solvents.[1,2] Lipids are also important in the function of the human body and also other biological systems.[2] Steroids are involved in the regulation of central metabolic pathways, energy metabolism, water and salt balance, and behavioral and cognitive functions.[3-11] Additionally, some steroids can serve as doping agents in sports due to their anabolic effects on muscle and muscle strength.[12-15] Vitamin D and its metabolites and oxysterols, on the other hand, are known to be involved in several diseases, such as bone metabolic diseases, certain cancers, autoimmune diseases and neurodegenerative diseases.[16-26] Therefore, these compounds are routinely analysed in biological samples in analytical laboratories all over the world. The concentrations of the selected compounds in biological samples can, however, be very low. Thus, in order to achieve correct information about these compounds in the human body, more sensitive and specific analytical methods are constantly needed.

This literature review focuses on the physiology of the selected lipids in the human body and it also summarizes the most important analytical methods used for the analysis of these compounds in biological samples.

1.1 SELECTED LIPIDS AND THEIR PHYSIOLOGY IN THE HUMAN BODY

1.1.1 STEROIDS

Steroid hormones are synthesized from cholesterol by a series of enzymatic reactions mainly in the adrenal glands and the gonads, and in the human feto-placental unit.[7,27,28] The steroid structure is based on a tetracyclic ring structure with different substituents that specify the steroid. Steroids are further divided into subclasses based on the number of carbon atoms present in the molecule: estrane (C_{18}), androstane (C_{19}), pregnane (C_{21}), cholane (C_{24}), and cholestane (C_{27}).[29] The structure of the subclasses and the steroid nomenclature are shown in Figure 1.

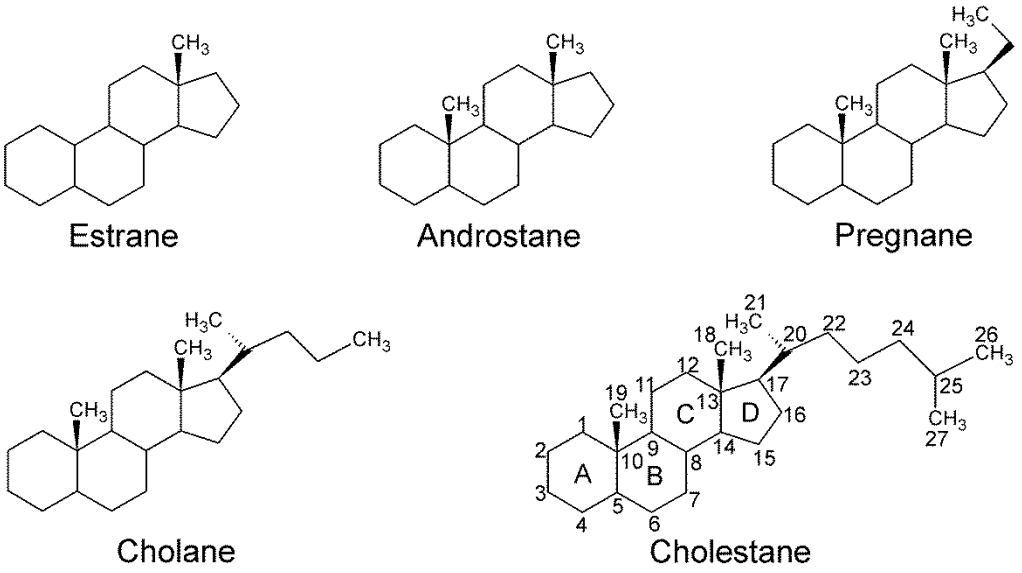


Figure 1 Structures of the steroid subclasses and the steroid nomenclature as identified by letters and numbers of carbon atoms. Adapted from Makin *et al*.[29]

It is common that the A or B ring of the steroid structure contains a double bond and that there are substituents including hydroxyl groups and/or carbonyl groups present at positions C-1, C-3, C-7, C-11, C-12, C-17, C-21, and/or C-25.[29] Steroids also have several chiral centers that lead to as many as 64 different stereoisomers with their substituents either above or below the molecular plane (α- or β-configuration).[29] It has been shown that the "bent" steroid ring structure associated with β-isomers may have significantly different biological effects in the human body compared to the more planar ring structure in α-isomers.[30,31]

Steroids can also be classified according to their biological activities. The most important steroid classes in this classification system are glucocorticoids, mineralocorticoids, sex steroids (androgens and estrogens) and vitamin D-derived hormones.[7,27,28] The physiological significance of steroids is exemplified by their participation in the regulation of various central metabolic pathways, including reproductive functions, energy metabolism, water and salt balance, and behavioral and cognitive functions.[3-11] The concentrations of many steroids vary considerably during different stages of life, which include age, stage of pregnancy, menstrual cycle, illness, and various drug treatments.[32-37] Consequently, the amount of steroids present in the human body is a valuable tool *inter alia* for clinical studies and for the diagnosis of endocrine disorders.[3,32-37]

Some steroids, namely the anabolic androgenic steroids (AAS), also serve as doping agents in sports due to their positive effect on muscle strength.[12-15] The use of these testosterone derivatives in sports was banned in 1976.[12,14] They are, however, also used in clinical practice for the treatment of different diseases and symptoms.[13,38]

1.1.2 VITAMIN D

Vitamin D exists naturally either as vitamin D_2 or as vitamin D_3 (Figure 2).[16,29] Mushrooms are a rich source of D_2, whereas fish are a rich source of D_3.[16,18,39-41] Vitamin D_3 is additionally biosynthesized in the skin from its precursor 7-dehydrocholesterol upon exposure to ultraviolet (UV) light (Figure 3). Vitamin D_2, on the other hand, is supplied only from dietary sources or from supplements.[42]

Vitamin D as such is biologically inactive and it is hydroxylated in the liver to 25-hydroxyvitamin D (25-OH-D), which is the major circulating form in the body.[17-19,29,39,40,43,44] The resulting 25-OH-D is then further metabolized in the kidney to the biologically active form 1α,25-dihydroxyvitamin D (1α,25-OH-D). Additionally numerous other metabolites of vitamin D have been well known for decades and the metabolism route has previously been thoroughly presented.[17,29,43,44] Figure 3 illustrates the biosynthesis of vitamin D_3 and the formation of the most important metabolites of vitamin D_3.

Vitamin D is well known for its importance in various functions in the human body and vitamin D deficiency or insufficiency has long been known to be a risk factor for bone metabolic diseases, such as rickets, osteomalacia, and osteoporosis.[16-19] It has also been shown, that vitamin D deficiency is involved in other diseases such as certain cancers (for example leukemia), autoimmune diseases and neurodegenerative diseases.[18,20-24]

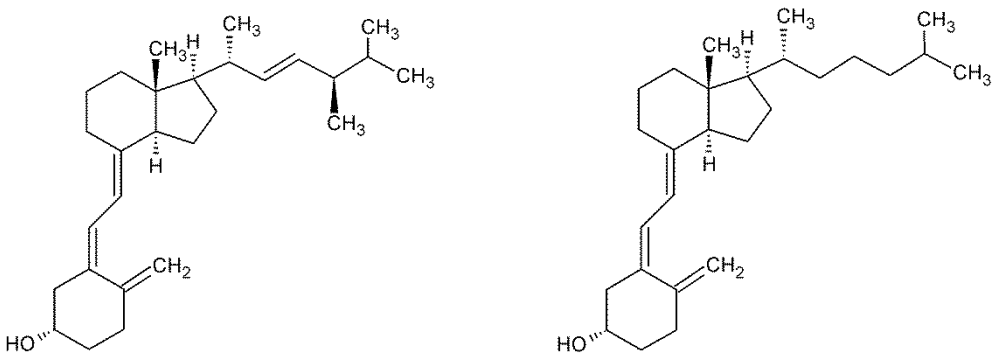


Figure 2 Structures of vitamin D_2 and D_3.

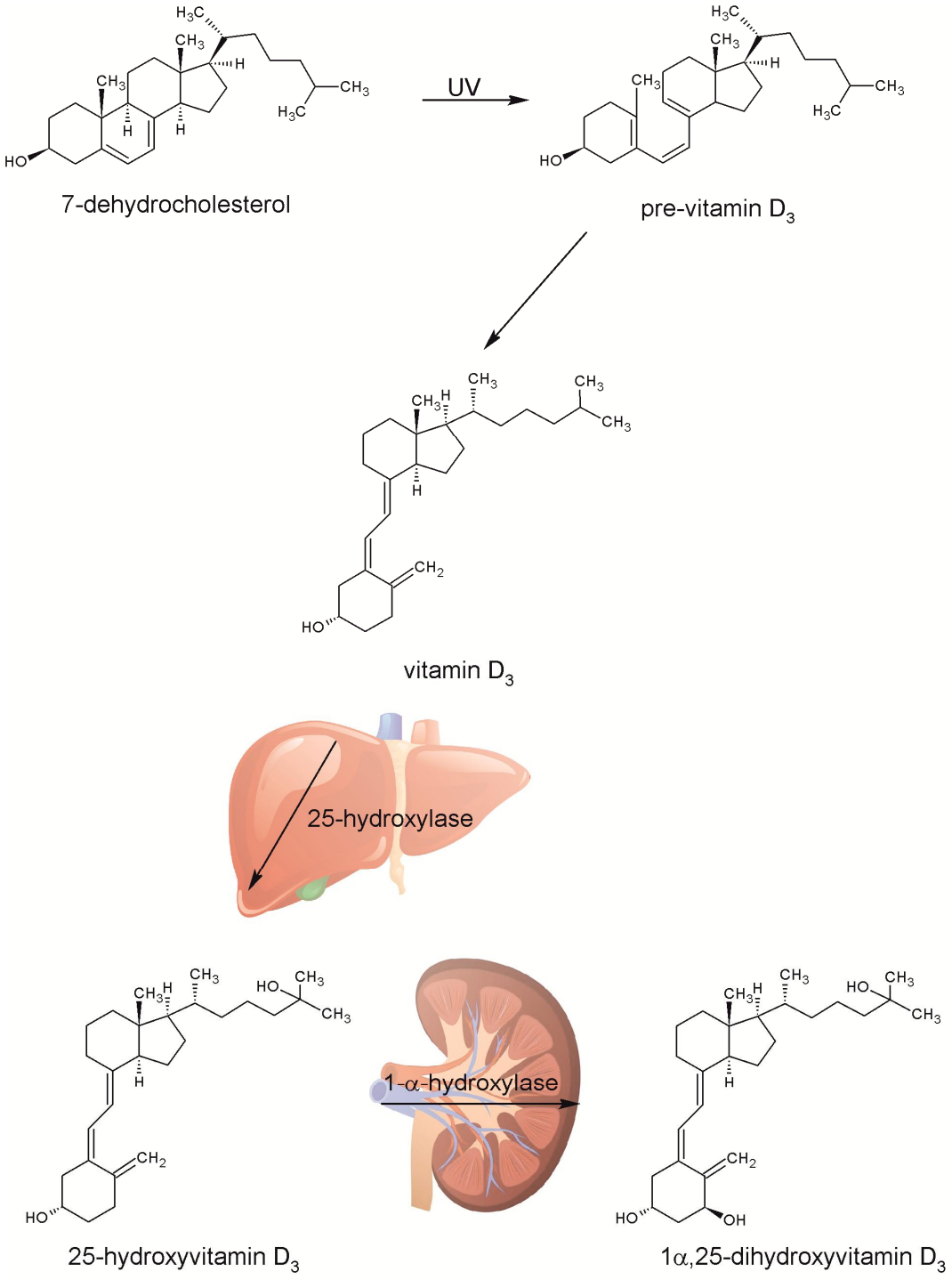


Figure 3 Biosynthesis of vitamin D_3 and formation of the most important metabolites of vitamin D_3.

1.1.3 OXYSTEROLS

Sterol lipids such as cholesterol and its derivatives are important components of membrane lipids.[45] Cholesterol is the precursor of the hormonal steroids and bile acids, and it can be found at high concentrations in the blood, brain and other steroidogenic tissues.[46,47] Cholesterol is also the starting material for the formation of oxysterols (Figure 4).[46,48,49] Oxysterols can be formed enzymatically by several different cytochrome P450 (CYP) enzymes in phase I metabolism of cholesterol and/or directly from cholesterol by reactive oxygen species.

Recently, oxysterols have been shown to be biologically active molecules.[26,46,50-52] Structurally different oxysterols have differing biological activity whereas the same oxysterols might even have different activities in different cells.[46] There is also evidence of changes in the concentrations of oxysterols as a result of neurodegenerative diseases.[25,26]

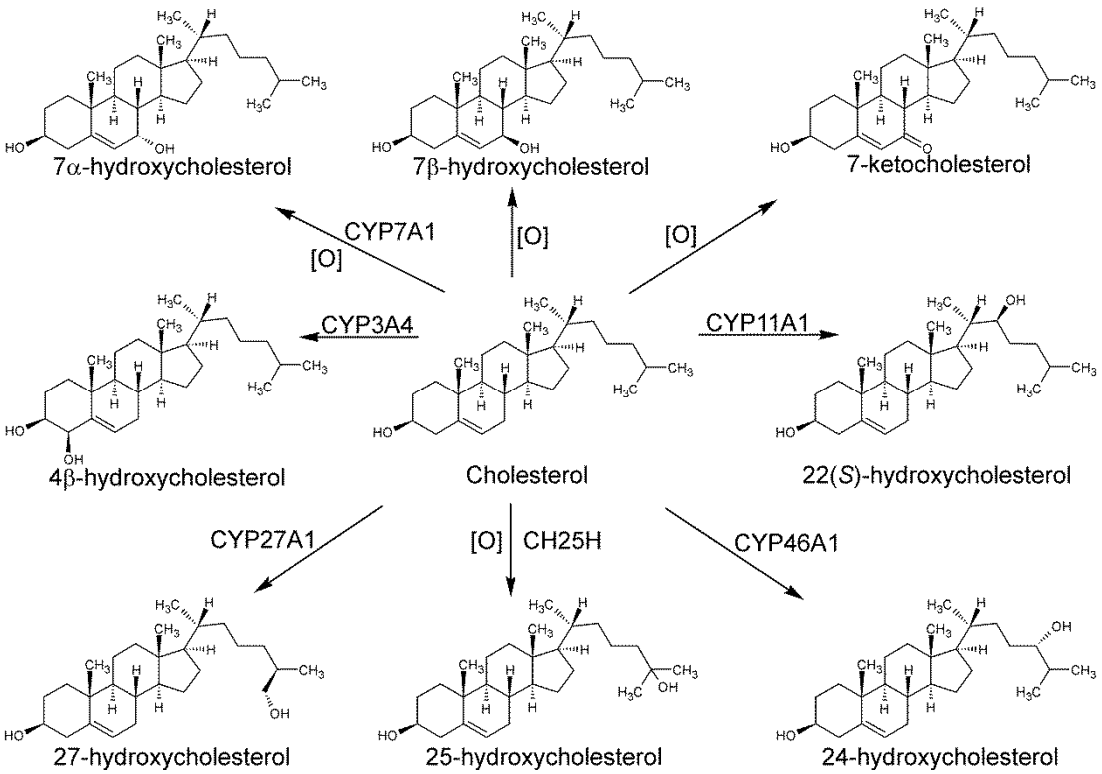


Figure 4 Formation of oxysterols from cholesterol by CYP enzymes or by reactive oxygen species [O]. Adapted from Griffiths *et al.*[46]

1.2 ANALYSIS OF STEROIDS, VITAMIN D AND OXYSTEROLS OF BIOLOGICAL SAMPLES

Steroids, vitamin D and oxysterols are present throughout the human body in a broad range of concentrations (from pg mL^{-1} to µg mL^{-1} depending on the compound).[53-55] In many cases the monitoring of these analytes can be highly significant such as in doping analysis and in the diagnostics of diseases. Therefore, the development of universal methods suitable for the simultaneous analysis of these compounds is of great importance. Methods developed for the analysis of these compounds have to be highly selective and sensitive, which sets a remarkable analytical challenge for analytical chemists. Also the tremendous amount of compounds and the complexity of different biological matrixes are challenging. Nevertheless, these classes of compounds have been detected and analysed in biological samples for a long time and the sample preparation procedures and analytical methods are constantly being updated.

1.2.1 SAMPLE PREPARATION

Analysis of the selected lipids (steroids, vitamin D and oxysterols) in biological samples usually requires some kind of sample pretreatment or sample cleanup before the actual analysis step. This is largely because there are a lot of compounds (e.g. other lipids) present in the biological matrix, and these might interfere with the determination of the targeted analytes. In some cases the compounds of interest exist in the biological samples mainly as their metabolites such as steroid glucuronides in urine samples,[56,57] or both as metabolites and as the native compounds. Thus, an enzymatic hydrolysis step is required prior to the actual sample cleanup in order to be able to analyse the total amount of these compounds present in the biological samples.

Sometimes a fast protein precipitation step might be enough for cleaning up the sample,[56,58-63] but usually the sample preparation procedures include the homogenization of the sample[46,64-66] and a liquid-liquid extraction (LLE)[46,56,59-61,64-69] and/or a solid-phase extraction (SPE)[46,56,59-61,64-71] step. LLE is still widely used, but nowadays other more efficient methods are often used.[68] In many cases, SPE is considered superior to LLE, because a better recovery of the analytes from the samples is usually achieved by SPE. SPE is also considered to be a more green method compared to LLE, as the consumption of organic solvents is usually lower than when SPE is used.

1.2.2 ANALYTICAL METHODS

The compounds included in the lipid classes investigated in this study affect many of the functions of the human body. Therefore, the determination of changes in the concentrations of these compounds in different biological samples can be used as a tool for example in the diagnostics of several diseases. Several analytical methods have been proposed for these analyses. Table 1 show some examples of previously proposed methods and this literature review focuses on the most important of the methods hitherto used.

1.2.2.1 Immunoassays

Immunological methods have for years been widely used in the analysis of steroids, vitamin D related compounds and oxysterols in biological samples.[72-76] The most common methods are based on radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA).[74-76] The advantage of using immunological methods is that they can allow for a high throughput of samples.[73,74] The suitability of immunoassays in the analysis of complicated biological samples is, however, questionable due to matrix effects and possible cross reactions.[72-74] Nevertheless, these methods are still in widespread use especially in clinical laboratories.[77,78]

1.2.2.2 Gas chromatography-mass spectrometry

There are some examples of analytical methods that utilize conventional gas chromatography (GC) with a flame ionization detector (FID) or an electron capture detector (ECD) for determining the concentration of selected lipids in biological samples.[46,79,80] However, since the coupling of gas chromatography to mass spectrometry (GC-MS) in the mid-1960s, this latter combination has been the preferred configuration for the analysis of the selected lipids.[29,67,79,81-89]

A GC-MS that uses electron ionization (EI) alleviates the concerns faced about the immunological methods since it provides good chromatographic resolution combined with high selectivity and sensitivity.[85,90-96] Unfortunately this method is time consuming, because most compounds require derivatization (in order to achieve evaporation of the analytes) prior to analysis.[67,79,81,82,90-92,94,96] The most commonly used procedure is to derivatize the hydroxyl groups of the analyte to trimethylsilyl (TMS) ethers.[67,82,90,91,94,97]

Despite the drawbacks of GC-EI-MS, this method is still considered as a reference method in steroid and oxysterol analyses.[67,92,98]

1.2.2.3 Liquid chromatography-mass spectrometry

The complicated sample pretreatment procedure needed for GC-MS analysis can be avoided by high performance liquid chromatography (HPLC) methods (with UV detection). These have been used in the analysis of lipids in biological samples.[44,67,74,80,82,98-101] Nowadays, the use of liquid chromatography-mass spectrometry (LC-MS) based methods have superseded HPLC-UV for the analysis of the selected lipids in biological samples.[44,46,73,96,100-104]

Electrospray ionization (ESI),[53,61,67,69,70,105] atmospheric pressure chemical ionization (APCI),[61,67,70,105-107] and atmospheric pressure photoionization (APPI)[62,70,105,108-111] have all been used in the analyses of these selected lipids in biological samples by LC-MS. ESI has proven to be an appropriate ionization technique for polar compounds.[112] However, the ionization efficiency of ESI for non-polar and neutral analytes can be poor.[112,113] Moreover, ESI is not applicable with non-polar mobile phase solvents and it can suffer from suppression caused by high ion concentrations. This limits the use of buffers and demands careful sample preparation before analysis.[112] These limitations entail that complementary ionization techniques such as APCI and APPI are used. APCI is more suitable for the analyses of less polar compounds compared to ESI. APCI also allows the use of polar and non-polar solvents, and tolerates higher electrolyte concentrations. APPI, on the other hand, was originally developed for the ionization of non-polar compounds, which can not be ionized by ESI or APCI.[113,114] APPI is also suitable for polar compounds and thus it can be considered a more general ionization method compared to ESI and/or APCI. In addition, suppression of ionization is significantly less with APCI and APPI than with ESI.[113]

The low ionization efficiency of APCI and ESI for many non-polar lipids requires different derivatization procedures to be utilized in order to enhance the ionization efficiency and hence the sensitivity of the methods.[53,61,64,67,69,107,115,116] The most commonly used derivatization reagents for the derivatization of steroids are hydroxylamine,[115,116] dansyl chloride,[53,107] 2-nitro-4-trifluoromethylphenylhydrazine (NFPH),[117] 2-hydrazino-1-methylpyridine (HMP),[64] and Girard P hydrazine (GP).[118] There are also some reports regarding the less common derivatization reagents, such as *p*-toluenesulfonyl isocyanate (PTSI), for steroids.[119,120] The derivatization reagents used for the derivatization of vitamin D related compounds, on the other hand, are slightly different from those used for steroids. The most common derivatization reagents are Cookson-type reagents.[54,61] Currently, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is the only reagent of this kind, which is commercially available. Oxysterols are also commonly derivatized for enhanced ionization efficiency.[67] The most common derivatization reactions for these steroid-like compounds include the following: the derivatization to picolinyl esters, to N,N-dimethyl glycine esters or to Girard hydrazones. Unfortunately, the derivatization step excludes the development of a universal analysis method suitable for all of these lipids, since the derivatization reaction is usually only possible on specific sites such as the *s-cis*-diene

structure in vitamin D related compounds or functional groups that include hydroxyl or carbonyl groups in steroids and oxysterols of the compound.[54,61,64,67,117]

1.2.2.4 Miniaturized analytical methods

A current trend in analytical chemistry is the miniaturization of conventional analysis techniques towards lab-on-a-chip devices.[121-124] The purpose of the miniaturization is to achieve rapid and sensitive analyses of very low sample amounts with minimal solvent and reagent consumption.[121,123,124] In addition, the possibility of automated analysis including all analysis steps i.e. sample preparation, separation, and detection being integrated on the same microchip is a goal that fascinates scientists.[121,122] In the past few years, the use of MS in microchip analysis has gained wide interest. This is based on the effort to couple micro- or nanofluidic separation systems to MS, and therefore the main interest is the miniaturization of ion sources.[122,125]

Until now, research has focused mainly on ESI, as the flow rates used in ESI are ideal for microfluidic devices.[122,125-127] Today, there are also some commercial microchips available for the coupling of low flow rate separation systems to MS.[125,128-133] One of these is the HPLC-Chip/MS system by Agilent Technolgies.[125,128] This system integrates a sample-enrichment column, a reversed phase LC separation column, and a nanoESI on a single polyimide chip, which eliminates the need for conventional LC connections.[128] The HPLC-Chip/MS system which is made by Agilent Technologies has most often been used for the analysis of proteins and peptides,[128,134-137] and to a lesser extent for the analysis of anthocyanins[138] and saccharides.[139,140] The Waters Corporation has also developed a microchip based LC-MS system called a NanoTile.[129-133] This relatively new device incorporates a reversed phase LC separation column, an electrospray ionization emitter and all the fluidic and electric connections needed. This new device has been used *inter alia* for the analysis of drugs[133] and proteins.[131,132] Both of these commercial systems have also been used for analyzing steroids in biological samples.[116,129,130]

The flow rates used with commercial APCI and APPI ion sources, on the other hand, are relatively high, typically above 50 µL min^{-1}. This makes these ion sources incompatible with low flow rate separations. A microfabricated heated nebulizer chip was introduced in 2004 in order to correspond to the need for low flow rate compatible APCI and APPI sources.[141,142] This new microchip was then successfully used as an APCI and APPI source. Since its original fabrication, the microchip has been refined,[143,144] and used for several other applications.[141,145-156] This microfabricated heated nebulizer provides flow rates down to 50 nL min^{-1}, excellent sensitivity, robust analysis, and efficient ionization for neutral and non-polar compounds including steroids.[141,145,146,148,150-153,156]

1.2.2.5 Ion mobility spectrometry mass spectrometry

The techniques of ion mobility spectrometry (IMS) and ion mobility spectrometry coupled to mass spectrometry (IMS-MS) have been known for decades and they have been used in numerous different applications and settings.[157-159] The separation that occurs in IMS-MS is based on the mass and the charge of the analyte in addition to other parameters.[157,160] These other parameters include the analyte collision cross section and the molecular interactions with the drift gases of different polarizabilities.

The IMS-MS technique also provides high sensitivity, specificity, and, most importantly, analysis times on a millisecond timescale. These features could be advantageous in the separation of steroid isomers, which is time consuming and laborious. The GC-MS and LC-MS techniques are currently used for the separation of the α/β isomers of steroids, but unfortunately these techniques require long chromatographic runs in order to achieve sufficient separation of the isomers. As a result, these otherwise highly sensitive and specific approaches lose their suitability especially for high throughput routine analysis and faster methods are needed in order to increase sample throughput and to decrease costs of analysis.

The development of IMS-MS equipment is becoming more popular among commercial instrument manufacturers and therefore the availability of this method is also increasing. IM techniques are also increasingly used in conjunction with LC-MS,[161-163] whereby IM provides an additional degree of separation and therefore significantly improved peak capacity and specificity. This can be particularly beneficial in the analysis of complex biological samples.[162-164] The recently introduced traveling wave ion mobility (TWIM) also provides a new mode of ion propulsion and separation for mobility experiments.[165] The TWIM device, when combined to MS (TWIM-MS), has been shown to be a powerful tool in bioanalysis. This is because it provides high ion transmission and good separation efficiency especially when using more polarizable and more massive drift-gases are used.[166,167]

Although IMS and IMS-MS are increasingly used in bioanalytics, there are only very few reports on the analysis of steroids that use these techniques.[119,162,163,168,169]

Table 1. *Examples of methods and achieved sensitivities used in the analysis of steroids, vitamin D and oxysterols. For abbreviations see chapter Abbreviations.*

Method	Compounds	Matrix	LOD/LOQ	Reference
LC-MS based methods	Endogenous steroids (23 analytes)	Urine	LOQ: 0.3 – 3 ng mL^{-1}	[170]
	Prohibited steroids (19 analytes)	Urine	LOQ: 2 – 15 ng mL^{-1}	[171]
	Prohibited doping substances (137 analytes)	Urine	LOD: 0.2 – 250 ng mL^{-1}	[172]
	Endogenous steroids (8 analytes)	Serum	LOQ: 8 pg mL^{-1}	[53]
	Vitamin D + metabolites (5 analytes)	Serum	LOQ: 1 – 2 ng mL^{-1}	[109]
	Vitamin D + metabolites (5 analytes)	Serum	LOQ: 25 pg mL^{-1}	[54]
	Vitamin D + metabolites (5 analytes)	Serum	LOQ: 0.2 – 0.6 ng mL^{-1}	[173]
	27-OH-Chl	Plasma	LOQ: 15 ng mL^{-1}	[108]
	4β-OH-Chl	Plasma	LOQ: 10 nM	[110]
	25-OH-D_3	Cerebrospinal fluid	LOQ: 0.04 nM	[174]
	25-OH-D_3	Serum	LOQ: 4 nM	[174]
GC-MS based methods	Endogenous steroids (46 analytes)	Brain	LOD: 0.005-1 ng g^{-1}	[93]
	Endogenous steroids (18 analytes)	Cerebrospinal fluid + serum	LOD: 0.04 – 1 pM	[55]
	Anabolic androgenic steroids (6 analytes)	Urine	LOD: 0.2 – 1 ng mL^{-1}	[146]
RIA	Vitamin D metabolites	Serum	LOD: 2 – 500 pg mL^{-1}	[75]
LC-IMS-MS based methods	Testosterone glucuronide + epitestosterone glucuronide	Urine	LOD: 0.7 – 7.4 ng mL^{-1}	[162]
	Anabolic androgenic steroids (5 analytes)	Urine	LOD: 50 – 500 pg mL^{-1}	[163]

2 AIMS OF THE STUDY

The overall aim of this research was to develop several different mass spectrometry based methods for the analysis of steroids, vitamin D related compounds and oxysterols, and to apply these developed methods to the analysis of biological samples.

The more detailed aims of the research papers, which are included in this thesis (I-IV) are the following:

- to evaluate the feasibility of the recently developed heated nebulizer microchip in APPI mode, when combined to capLC-MS/MS, in the analysis of anabolic steroids in human urine samples (I)

- to compare a commercially available HPLC-Chip/ESI/MS system with a conventional LC-ESI/MS in the analysis of oxime derivatized steroids and to apply the microchip based system to the analysis of non-derivatized steroids in mouse plasma samples (II)

- to apply ESI-TWIM-MS for separating selected non-derivatized and *p*-toluenesulfonyl isocyanate derivatized steroid isomers using two different drift gases (N_2 and CO_2) at different pressures (III)

- to develop and to compare two highly sensitive and selective UHPLC-APPI-MS methods for the analyses of vitamin D related compounds and oxysterols in mouse brain samples and cell line samples (IV)

3 MATERIALS AND METHODS

This section briefly describes the chemicals, samples and sample pretreatment procedures, instrumentation and analytical methods used in this study. More detailed descriptions can be found in publications I-IV.

3.1 CHEMICALS

The solvents used in this study are listed in Table 2. All solvents were of HPLC grade unless otherwise stated.

Table 2. *Solvents (with abbreviations) used in the study.*

Solvent	Manufacturer/Supplier	Publication
Acetonitrile (ACN)	Rathburn Chemicals Ltd., Walkerburn, Scotland	II
	Sigma-Aldrich, St. Louis, MO, USA	III
	Sigma-Aldrich, Steinheim, Germany (LC-MS grade)	IV
Dichloromethane (DCM)	Sigma-Aldrich, Steinheim, Germany	IV
Diethyl ether (freshly distilled)	VWR, Espoo, Finland	I
Isopropanol (IPA) LC-MS grade	Sigma-Aldrich, Steinheim, Germany	IV
Methanol (MeOH)	Mallinckrodt Baker B.V., Deventer, The Netherlands	I, II
	Sigma-Aldrich, Steinheim, Germany (HPLC grade and LC-MS grade)	II, IV
	J.T. Baker, Mexico City, Mexico	III
Methyl tertiary-butyl ether (MTBE)	Sigma-Aldrich, Steinheim, Germany	II
Toluene (APPI dopant)	Sigma-Aldrich, St. Louis, MO, USA	I
	Sigma-Aldrich, Steinheim, Germany	IV
Water (milli-Q, H_2O)	Millipore, Molsheim, France	I, II, IV
	Millipore, Billerica, MA, USA	III

The reagents used in this study are listed in Table 3. All reagents were of reagent grade unless otherwise stated.

Table 3. *Reagents (with abbreviations) used in the study.*

Reagent	Manufacturer/Supplier	Publi-cation
Ammonium acetate (NH_4Ac)	Sigma-Aldrich, Steinheim, Germany	II
Ammonium hydroxide (NH_4OH)	Acros Organics, New Jersey, USA	III
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany	II
Formic acid (HCOOH)	Sigma-Aldrich, Steinheim, Germany (HPLC grade) Acros Organics, New Jersey, USA (analytical grade)	II III
β-Glucuronidase (from *Helix Pomatia,* type HP-2)	Sigma-Aldrich, St. Louis, MO, USA	I
Hydroxyl amine hydrochloride (HPLC grade)	Sigma-Aldrich, Steinheim, Germany	II
Potassium acetate	Mallinckrodt Baker B.V., Deventer, The Netherlands	I
Potassium carbonate	Mallinckrodt Baker B.V., Deventer, The Netherlands	I
Sodium hydrogen carbonate	Mallinckrodt Baker B.V., Deventer, The Netherlands	I
p-toluenesulfonyl isocyanate (PTSI, 96%)	Sigma-Aldrich, St. Louis, MO, USA	III

The standards (with abbreviations) used in this study are listed in Table 4. The structures of the studied compounds are shown in publications I-IV.

Table 4. *Standards (with abbreviations) used in the study.*

Standard	Manufacturer/Supplier	Publi-cation
Aldosterone (A)	Sigma-Aldrich, Steinheim, Germany	II
Androstenedione (AN)	Steraloids Inc., Newport, RI, USA Sigma-Aldrich, Steinheim, Germany	II
Androsterone	Sigma-Aldrich, Steinheim, Germany (AND) Sigma-Aldrich, St. Louis, MO, USA (3α-AN)	II III
Cholecalciferol (D_3)	Enzo Life Sciences Inc., Farmingdale, NY, USA	IV
d3-Cholecalciferol (d_3-D_3)	Sigma-Aldrich, Steinheim, Germany	IV
Corticosterone (CORT)	Sigma-Aldrich, Steinheim, Germany	II
Cortisone (CS)	Sigma-Aldrich, Steinheim, Germany	II
7-Dehydrocholesterol (7-DHYD)	Sigma-Aldrich, Steinheim, Germany	IV
Dehydroepiandrosterone (DHEA)	Sigma-Aldrich, Steinheim, Germany	II
11-Deoxycortisol (11-DC)	Sigma-Aldrich, Steinheim, Germany	II
Desmosterol (DESMO)	Avanti Polar Lipids Inc., Alabaster, Al, USA	IV
Dihydrotestosterone (DHT)	Sigma-Aldrich, Steinheim, Germany	II
1α,25-Dihydroxycholecalciferol (1**α**,25-D_3)	Sigma-Aldrich, Steinheim, Germany	IV
17-Epimetandienone (17MDN)	United Medix Laboratories Ltd., Helsinki, Finland	I
Epitestosterone (17**α**-T)	Sigma-Aldrich, St. Louis, MO, USA	III
Ergocalciferol (D_2)	Sigma-Aldrich, Steinheim, Germany	IV
α-Estradiol (**α**-ES)	Sigma-Aldrich, St. Louis, MO, USA Sigma-Aldrich, Schnelldorf, Germany	III
β-Estradiol (**β**-ES)	Sigma-Aldrich, St. Louis, MO, USA	III
Estrone (ES)	Sigma-Aldrich, Steinheim, Germany	II
Hydrocortisone (HC)	Sigma-Aldrich, Steinheim, Germany	II
25-Hydroxycholecalciferol (25-OH-D_3)	Sigma-Aldrich, Steinheim, Germany	IV
7**α**-Hydroxycholesterol (7**α**-OH-Chl)	Fountain Limited, Naxxar, Malta	IV
7**β**-Hydroxycholesterol (7**β**-OH-Chl)	Fountain Limited, Naxxar, Malta	IV

Table 4 continued *Standards (with abbreviations) used in the study.*

Standard	Manufacturer/Supplier	Publi-cation
22(*S*)-Hydroxycholesterol (22-OH-Chl)	Fountain Limited, Naxxar, Malta	IV
24(*S*)-Hydroxycholesterol (24-OH-Chl)	AH Diagnostics Oy, Helsinki, Finland	IV
27-Hydroxycholesterol (27-OH-Chl)	Santa Cruz Biotechnology Inc., Heidelberg, Germany	IV
d_6-27-Hydroxycholesterol (d_6-27-OH-Chl)	Avanti Polar Lipids Inc., Alabaster, Al, USA	IV
17-Hydroxypregnenolone (17-OH-PREG)	Sigma-Aldrich, Steinheim, Germany	II
17-Hydroxyprogesterone (17-OH-PROG)	Sigma-Aldrich, Steinheim, Germany	II
7-Ketocholesterol (7-OXO)	Avanti Polar Lipids Inc., Alabaster, Al, USA	IV
Metabolite of nandrolone (NANm)	United Medix Laboratories Ltd., Helsinki, Finland	I
Methyltestosterone (MTS)	United Medix Laboratories Ltd., Helsinki, Finland	I
Nandrolone (NAN)	United Medix Laboratories Ltd., Helsinki, Finland	I
Pregnenolone (PREG)	Sigma-Aldrich, Steinheim, Germany	II
Progesterone (PROG)	Sigma-Aldrich, Steinheim, Germany	II, IV
Testosterone	Sigma-Aldrich, Steinheim, Germany (T) Sigma-Aldrich, St. Louis, MO, USA (17β-T)	II III
d_3-Testosterone (d_3-T)	Sigma-Aldrich, Steinheim, Germany	II
Trans-androsterone (3β-AN)	Sigma-Aldrich, St. Louis, MO, USA	III

Stock solutions of the standards (1 mg mL^{-1}, 5 mM or 1 mM) were prepared in MeOH or in ACN depending on the solubility of the compound. Further dilutions of the stock solutions were made by using different solvents depending on the final use of the solution. More detailed information can be found in studies I-IV.

The samples used in this study are listed in Table 5. Detailed information about the samples can be found in publications I, II, and IV.

Table 5. *Samples and sample matrix used in the study.*

Sample/Matrix	Manufacturer/Supplier	Publi-cation
Mouse plasma samples	University of Turku, Central Animal Laboratory, Turku, Finland	II
Pooled drug-free human urine	United Medix Laboratories Ltd., Helsinki, Finland	I
Artificial plasma matrix: Ringer's solution + 4% (w/v) BSA	University of Helsinki, Faculty of Pharmacy, Division of Pharmaceutical Chemistry, Helsinki, Finland	II
WT SH-SY5Y human neuroblastoma cell line samples	University of Helsinki, Faculty of Pharmacy, Division of Pharmacology and Toxicology, Helsinki, Finland	IV
A30P and A53T α-syn cell line samples	University of Helsinki, Faculty of Pharmacy, Division of Pharmacology and Toxicology, Helsinki, Finland	IV
Mouse brain samples	University of Helsinki, Faculty of Pharmacy, Division of Pharmacology and Toxicology, Helsinki, Finland	IV

3.2 SAMPLES AND SAMPLE PRETREATMENT PROCEDURES

Several different biological sample types were used in this study in order to prove the utility of the developed methods for lipid analysis. The samples were provided as collected, and therefore sample pretreatment procedures were used as well.

3.2.1 URINE SAMPLES

Pooled drug-free human urine was used to test the feasibility of the new capLC-μAPPI-MS/MS method for the analysis of anabolic steroids in human urine samples (I). A 2.5 mL aliquot of pooled drug-free human urine sample was spiked to a concentration of 100 ng mL^{-1} MTS (internal standard). The urine was then buffered to pH 5.0 with 0.2 M potassium acetate buffer and enzymatically hydrolyzed by 50 mL of β-glucuronidase at 55 °C for 2 h. 250 mg of a solid mixture of sodium hydrogen carbonate and potassium carbonate (2:1, w/w) was added and the samples were extracted using diethyl

ether. After centrifugation, the organic layer was separated and evaporated to dryness. Finally, the samples were dissolved in 100 µL of 15% MeOH and analyzed by capLC-µAPPI-MS/MS.

3.2.2 MOUSE PLASMA SAMPLES

Mouse plasma samples were analysed by the HPLC-Chip/MS system in order to prove the feasibility of a newly developed method in the analysis of non-derivatized steroids (II). The samples were obtained from mice with a C57B1/129 hybrid background. All animal handling was conducted in accordance with the Finnish Animal Ethics Committee and the Institutional animal care policies of the University of Turku, which fulfill the requirements of the NIH Guide on animal experimentation.

Steroids were extracted from the mouse plasma samples using LLE with MTBE. Briefly, the procedure was as follows: 1 mL of MTBE was added to a 150 µL aliquot of mouse plasma. The sample was vortex mixed for one minute and centrifuged (at 2000 rpm for 5 min). The MTBE phase was filtered into a sample vial through a 0.2 µm syringe filter (Millipore, Bedford, MA, USA) and evaporated to dryness. Finally, the samples were reconstituted in 50 µL of 5% NH_4Ac (50 mM, pH 4.5 with acetic acid):MeOH:ACN (1:3:6, v/v) and vortex mixed just before analysis by the HPLC-Chip/MS system.

3.2.3 MOUSE BRAIN SAMPLES

Three month old male NMRI-mice were used for the analysis of oxysterols and vitamin D metabolites in brain samples by UHPLC-APPI-MS (IV). The protocol for animal handling was accepted by the Animal Experiments Board of Finland and experiments were performed according to Finnish legislation.

The mice were anaesthetized using sodium pentobarbital (100 mg kg^{-1}, i.p.) as the agent and transcardially perfused with phosphate buffered saline (PBS) for four minutes to purge all the blood out of the brain. Afterwards the brains were removed from the scull, frozen on dry ice and stored at -80 ºC until assayed.

The intact mice brains were weighed and cut into four parts. A 2.5 µL aliquot of a 1 µg mL^{-1} standard working solution containing the internal standards d_3-D_3 and d_6-27-OH-Chl was added to each brain part. The samples were homogenized and extracted by LLE. The procedure was as follows: 0.5 mL of a DCM:MeOH mixture (1:1, v/v) was added to the samples, which were homogenized using ultrasonication with a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT, USA) in an ice bath for 1 min. The ultrasonication had an amplitude and efficiency of 90% and 2, respectively. The samples were centrifuged (at 13200 rpm for 5 min), the supernatants were removed and the procedure was repeated. After the second extraction process, the four supernatants that originated from each intact mouse brain were combined into one sample for that respective brain, which was then

evaporated to dryness. The samples were reconstituted in 100 µL of MeOH, centrifuged (at 13200 rpm for 5 min) and the supernatants were moved to a vial with a glass insert right before analysis by UHPLC-APPI-MS.

3.2.4 CELL LINE SAMPLES

The wild type (WT) SH-SY5Y human neuroblastoma cell line was purchased from ATCC (LGC Standards, Product # CRL-2266, Middlesex, UK) and cultured as described by Myöhänen *et al.* [175] Stable cell lines that express A30P and A53T α-syn were generated using a lentiviral vector as described by Gerard *et al.*,[176] and the transfected cells were cultured as described by Myöhänen *et al.*[175]

The cell lines for the analysis by UHPLC-APPI-MS (IV) were used at passages 3 to 15 and grown at 37 ºC and 5% CO_2 in a humidified atmosphere. For oxidative stress, 1×10^6 cells were seeded in T25-flasks and allowed to grow overnight. Thereafter, the aggregation process of α-syn was induced by adding 100 µM H_2O_2 and 10 mM $FeCl_2$ in cell culturing medium for three days as previously described.[175,176] All cells (non-stressed and stressed) were homogenized as described by Myöhänen *et al.* using 0.1 M Na-K phosphate buffer (pH 7.0).[175] The homogenates were centrifuged (at 16000 *g*, 4 ºC for 20 min), and thereafter the supernatants and pellets were separated and stored at -80 ºC until assayed.

A 10 µL aliquot of the 1 µg mL^{-1} standard working solution that contained the internal standards d_3-D_3 and d_6-27-OH-Chl was added to the cell pellet. The samples were homogenized and extracted by LLE with 0.5 mL of a DCM:MeOH mixture (2:1, v/v) following the same procedure as was used for the brain samples. Finally, the supernatants were combined, evaporated to dryness and reconstituted in 100 µL of MeOH before analysis by UHPLC-APPI-MS.

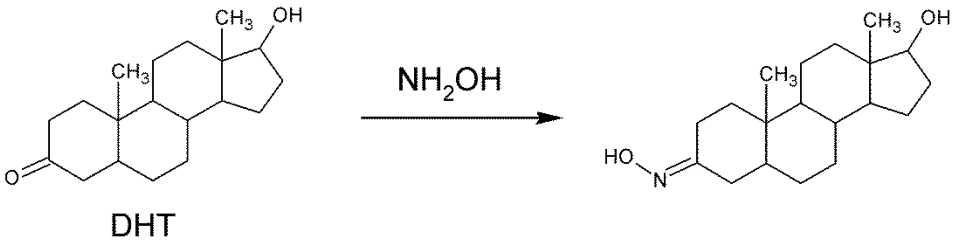
3.3 DERIVATIZATION OF STEROIDS

Steroids are relatively nonpolar compounds and therefore not very well ionizable by ESI. Therefore, it is quite common to derivatize these compounds before analysis in order to enhance the ionization efficiency and thereby the sensitivity of the method. In this study two different derivatization methods were used (II and III), but the purposes were quite different. In publication II, derivatization was used in order to enhance the ionization efficiency in ESI. In publication III, on the other hand, derivatization was used in order to increase the difference of either the strength of the ion/molecule interactions with the drift gas and/or the difference in collision cross sections of the analytes. The purpose of this was to improve the separation efficiency of the steroid isomers in the IMS-MS experiments.

3.3.1 OXIME DERIVATIZATION

Oxime derivatization was used in publication II in order to enhance the ionization efficiency in ESI when comparing a commercially available HPLC-Chip/ESI/MS system to a conventional LC-ESI/MS. An example of the derivatization reaction is shown in Figure 5.

The ketosteroids in the samples were derivatized by adding 600 µL of hydroxylamine solution (NH_2OH, 100 mM in 50% MeOH) to each sample and heating at 60 ºC for 30 min in a ULE500 laboratory oven (Memmert, Schwabach, Germany). Thereafter, the samples were analysed.



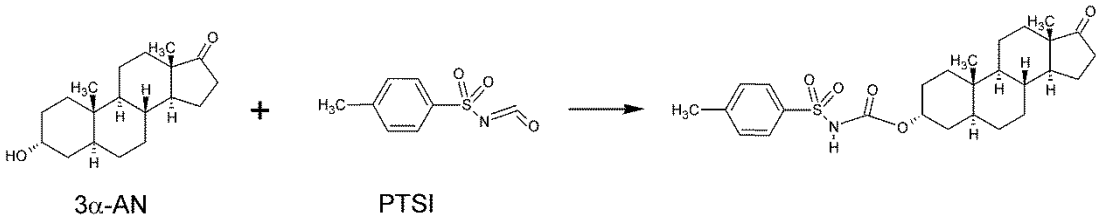
DHT

Figure 5 Example of the oxime derivatization reaction for DHT.

3.3.2 *P*-TOLUENESULFONYL ISOCYANATE DERIVATIZATION

In publication III steroid isomers were subjected to derivatization by *p*-toluenesulfonyl isocyanate (PTSI). Figure 6 shows an example of the derivatization reaction. In this case, the derivatization was not related to enhancing the ionization efficiency. The purpose was instead to increase the difference of either the strength of the ion/molecule interactions with the drift gas and/or the difference in collision cross sections and thus to improve the separation efficiency of the steroid isomers in the TWIM-MS analysis.

In order to derivatize the samples, 100 µL of the working standard solution of each analyte was transferred to a glass vial, and the solution was evaporated to dryness. 100 µL of ACN and 20 µL of PTSI (1:10, v/v in ACN) were added to the vial. The sample mixture was vortexed at ambient temperature for 2 min and the derivatization reaction was stopped by adding 20 µL of H_2O and vortexing for 30 s. After this, 860 µL of MeOH, including 0.1% NH_4OH, was added to the solution.



3α-AN PTSI

Figure 6 Example of the *p*-toluenesulfonyl isocyanate derivatization reaction.

The derivatization of the hydroxyl group at position C17 of α-ES was confirmed by nuclear magnetic resonance (NMR) using ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR analysis. A chemical synthesis was performed in larger scale before the actual analysis step in order to achieve the amount of product needed for NMR analysis. More detailed information can be found in Electronic Supplementary Information 1 in publication III.

3.4 INSTRUMENTATION AND ANALYTICAL METHODS

The analyses were performed on several different mass spectrometers with variable additional instruments. New analytical methods were developed with the purpose of assessing novel methods for the analysis of the selected lipids. Table 6 briefly outlines the instrumentation used in this study. More detailed information can be found in studies I-IV.

The home-built mass spectrometer ion source, µAPPI, used in publication I for analyzing anabolic steroids in urine samples, was originally described in 2004 by Kauppila *et al.*[142] Since that first introduction, some improvements have been implemented, but the basic functions remain the same. A schematic view of the µAPPI setup is presented in Figure 7. The fabrication of the microchips used for the ionization has been presented in more detail by Saarela *et al*.[144] Publication I also contain detailed descriptions on the positioning of the microchip in front of the mass spectrometer.

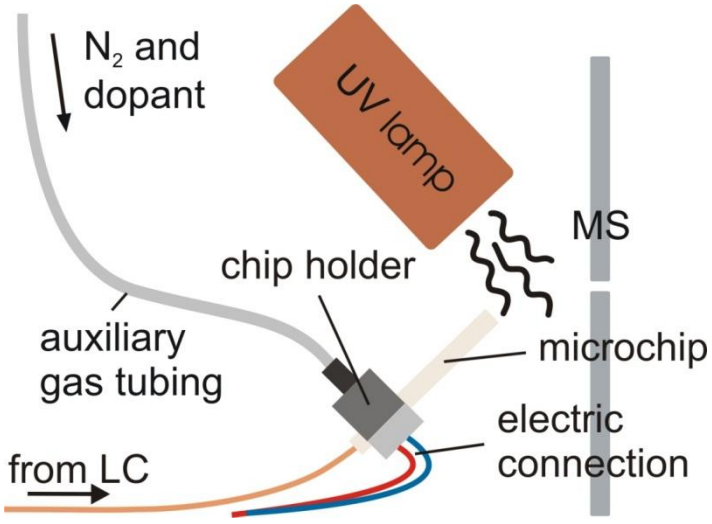


Figure 7 Schematic view of the µAPPI setup in front of the mass spectrometer

Tables 7-10 briefly summarize the analytical methods used and developed in this study. Detailed information can be found in studies I-IV.

Table 6. *Instrumentation used in this study.*

Instrument	Manufacturer	Note	Publi-cation
PE Sciex API 300 triple quadrupole	MDS Sciex, Concord, Canada	A *xyz* positioning stage was used for positioning the microchip in front of the mass spectrometer	I
75-72 nitrogen generator	Whatman Inc., Haverhill, MA, USA	Used for generating purified nitrogen to be used as a curtain and nebulizer gas	I
Krypton discharge UV lamp (PKS 100)	Cathodeon, Cambridge, UK	Used for the photoionization of the analytes using 10 eV photon energy	I
APPI power source	University of Groningen, Groningen, The Netherlands	Custom-made at the Electronics Facility and Mechanical Workshop	I
dc power supply (IPS-603)	RS Components, Corby, UK	Used for heating of the microchip at 3.5 W of power (corresponding to approximately 350 °C)	I
Mass flow controller (GCF17)	Aalborg, Orangeburg, NY, USA	Used for regulating the nebulizing gas flow (150 mL min^{-1}) into the microchip	I
Syringe pump (PHD 2000)	Harvard Apparatus, Holliston, MA, USA	Used for dopant (toluene) delivery at a flow rate of 2 µL min^{-1} and for the optimization of the mass spectrometer voltages for each analyte	I
CapLC (1100 series)	Agilent Technologies, Waldbronn, Germany	Symmetry Shield column (100 × 0.3 mm i.d., particle size 3.5 µm), Waters, Milford, MA, USA Only the pump and degasser was used in publications II and IV	I II, IV

Table 6 continued *Instrumentation used in this study.*

Instrument	Manufacturer	Note	Publi-cation
Agilent 6410 triple quadrupole	Agilent Technologies, Waldbronn, Germany	The mass spectrometer was used with the HPLC-Chip/MS interface	II
Parker Balston N2-22 nitrogen generator	Parker Hannifin, Haverhill, MA, USA	Used for generating purified nitrogen to be used as a drying and collision gas	II
Syringe pump	Cole-Parmer, Vermon Hills, IL, USA	Used for the optimization of the ionization and mass spectrometric conditions for each analyte when using the chip cube	II
RRLC (1200 series)	Agilent Technologies, Waldbronn, Germany	Zorbax SB-C18 column (50 × 2.1 mm i.d., particle size 1.8 µm), Agilent Technologies, Waldbronn, Germany	II
NanoLC (1200 series)	Agilent Technologies, Waldbronn, Germany	The nanopump (and degasser) and the microwell-plate autosampler were used for sample delivery only.	II
Chip cube (HPLC-Chip/MS interface)	Agilent Technologies, Santa Clara, CA, USA	The polymeric chip (either HPLC or MS calibration and diagnostic chip) is inserted into the chip cube, which includes a miniature camera for spray visualization	II
MS calibration and diagnostic chip	Agilent Technologies, Santa Clara, CA, USA	Used for the optimization of ionization and mass spectrometric conditions for each analyte	II
HPLC chip	Agilent Technologies, Santa Clara, CA, USA	Integrates a 40 nL enrichment column packed with 5 µm Zorbax 300 SB-C18 particles, a 50 µm (d) × 75 µm (w) × 43 mm (l) RP-LC separation column packed with 5 µm Zorbax 300 SB-C18 particles, and a nanospray emitter (50 µm i.d.)	II

Table 6 continued *Instrumentation used in this study.*

Instrument	Manufacturer	Note	Publi-cation
Waters Synapt HDMS	Waters, Manchester, UK	Including a hybrid quadrupole/traveling wave ion mobility/orthogonal acceleration time-of-flight geometry	III
Waters ACQUITY UPLC® I Class	Waters, Milford, MA, USA	ACQUITY UPLC® BEH C18 column (100 × 2.1 mm i.d., particle size 1.7 µm), Waters, Milford, MA, USA	IV
Waters ACQUITY UPLC®	Waters, Milford, MA, USA	ACQUITY UPLC® HSS C18 column (100 × 2.1 mm i.d., particle size 1.8 µm	IV
Waters Synapt G2-S HDMS (Q-TOF)	Waters, Wilmslow, UK	Interfaced with a Zspray™ Atmospheric Pressure Photoionization/Atmospheric Pressure Chemical Ionization (APPI/APCI) dual source, Waters, Milford, MA, USA	IV
Waters® Xevo™ TQ-S MS (triple quadrupole)	Waters, Milford, MA, USA	Interfaced with a Zspray™ Atmospheric Pressure Photoionization/Atmospheric Pressure Chemical Ionization (APPI/APCI) dual source, Waters, Milford, MA, USA	IV

Table 7. *Analytical methods used and developed for the analysis of anabolic steroids in human urine samples by capLC-µAPPI-MS/MS (I).*

Analytes	Molecular weight	Mass spectrometric conditions	Chromatographic conditions			
			Mobile phase	Gradient		
				Time (min)	*Org %*	
17MDN	300	Positive ion mode	A: H_2O:MeOH (95:5, v/v)			*Injection volume:* 3 µL
				0-2	15-75	
MTS	302	µAPPI		2-13	75-95	
				13-15	95-100	
NAN	274	Selected reaction monitoring (SRM)	B: MeOH	15-30	100	*Flow rate:* 6 µL min^{-1}
				30-30.5	100-15	
NANm	276			30.5-60.5	15	

Table 8. *Analytical methods used and developed for the analysis of oxime derivatized and non-derivatized steroids by the conventional LC-MS system and by the HPLC-Chip/MS system (II).*

Analytes	Molecular weight	Mass spectrometric conditions	Chromatographic conditions			
Oxime deriv.			*Conventional LC-MS system*			
			Mobile phase	*Gradient*		
AN	286			Time (min)	Org %	
						Injection volume:
AND	290			0-0.7	60	40 µL
				0.7-5	60-90	
DHEA	288			5-5.4	90	*Flow rate:*
		Positive ion mode		5.4-5.41	90-60	0.2 mL min^{-1}
DHT	290		A: H_2O + 0.025%	5.41-7	60	
			(v/v) HCOOH			
ES	270	ESI		*HPLC-Chip/MS system*		
			B: MeOH + 0.025%	*Gradient*		*Sample loading:*
17-OH-PREG	332		(v/v) HCOOH	Time (min)	Org %	3% B, flow rate
		SRM				4 µL min^{-1}
PREG	316			0-0.3	5-50	
				0.3-7	50-85	*Injection volume:*
17-OH-PROG	330			7-7.1	85-5	0.5 µL
				7.1-20	5	
T	288					*Flow rate:*
						0.3 µL min^{-1}

Table 8 continued *Analytical methods used and developed for the analysis of oxime derivatized and non-derivatized steroids by the conventional LC-MS system and by the HPLC-Chip/MS system (II).*

Analytes	Molecular weight	Mass spectrometric conditions	Chromatographic conditions			
Non-deriv.			*HPLC-Chip/MS system*			
			Mobile phase	*Gradient*		
A	360					
AN	286					
			A: NH_4Ac	Time (min)	Org %	*Sample loading:*
CORT	346	Positive ion mode	(50 mM, pH 4.5):H_2O			3% B, flow rate
			(1:9, v/v)	0-0.3	5-40	4 µL min^{-1}
CS	360			0.3-3.9	40-40.2	
		ESI		3.9-4	40.2-76	*Injection volume:*
11-DC	346		B: NH_4Ac	4-6.6	76-80	0.75 µL
			(50 mM, pH 4.5)	6.6-7.8	80-100	
HC	362	SRM	MeOH:ACN	7.8-7.9	100-5	*Flow rate:*
			(1:3:6, v/v/v)	7.9-32.9	5	0.3 µL min^{-1}
PROG	314					
T	288					

Table 9. *Analytical methods used and developed for the separation of steroid isomers by TWIM-MS (III).*

Analytes	Molecular weight	MS conditions	TWIM-MS conditions			
			Non-derivatized analytes			Derivatized analytes
		Direct infusion:	*Sample solvent*	*Ion mode*	*Drift gas pressures*	*Sample solvent:* MeOH + 0.1% NH_4OH
3α-AN	290	Concentration: 10 µg mL^{-1}	MeOH + 0.1% HCOOH	ESI+	N_2: 2.0 mbar CO_2: 1.0 mbar	*Ion mode:* ESI-
3β-AN	290					
α-ES	272	Flow rate: 30 µL min^{-1}	MeOH + 0.1% NH_4OH	ESI-	N_2: 2.0 mbar CO_2: 1.3 mbar	*Drift gas pressures:* N_2: 2.0 mbar, 2.5 mbar, 3.0 mbar CO_2: 1.0 mbar, 1.3 mbar
β-ES	272					
17**α**-T	288	MS scan	MeOH + 0.1% HCOOH	ESI+	N_2: 2.0 mbar CO_2: 1.3 mbar	
17**β**-T	288					

Table 10. *Analytical methods used and developed for the analysis of vitamin D related compounds and oxysterols in brain samples and in cell line samples by UHPLC-APPI-MS (IV).*

Analytes	Molecular weight	MS conditions	Chromatographic conditions		
			Mobile phase	Gradient	
				Time (min) *Org %*	
D_3	384	Positive ion mode APPI SRM (TQ-S) Sensitivity mode & MS^e-mode (Q-TOF)	A: H_2O B: MeOH		*Injection volume:* 5 µL (TQ-S) 7.5 µL (Q-TOF) *Flow rate:* 0.3 mL min^{-1} *Dopant (TQ-S):* toluene flow rate 20 µL min^{-1} *Lock mass and dopant (Q-TOF):* 0.1 µg mL^{-1} PROG in toluene flow rate 30 µL min^{-1}
d_3-D_3	387			0-14 80-100	
D_2	396			14-17 100	
1α,25-OH-D_3	416			*Column wash:* 5 min 10% DCM in MeOH	
25-OH-D_3	400				
d_6-27-OH-Chl	408				
27-OH-Chl	402				
24-OH-Chl	402				
22-OH-Chl	402			*Column re-equilibration:* 6 min 80% B	
7α-OH-Chl	402				
7β-OH-Chl	402				
DESMO	384				
7-OXO	400				
7-DHYD	384				

4 RESULTS AND DISCUSSION

The need for new fast, robust and highly sensitive methods in lipid analysis is continuously growing. The variety of compounds of interest is also increasing constantly. In this study new methods for lipid analysis were developed and the feasibilities of these methods were tested in the analyses of several biological samples. This section of the thesis summarizes the main results of the work. More details regarding the results can be found in the original publications (I-IV).

4.1 CAPLC/MICROCHIP-APPI/MS/MS IN THE ANALYSES OF ANABOLIC STEROIDS OF URINE SAMPLES

The aim of the first part of this study was to develop a capLC-μAPPI-MS/MS method for the analysis of anabolic steroids in human urine samples. For this purpose, an in-house built microchip was used. This heated nebulizer microchip had hitherto been used for several applications, but no LC based methods for biological samples had been developed. Nevertheless, the technique of μAPPI seemed promising for this purpose since it would allow smaller consumption of organic solvents and it would also provide the possibility of lower flow rates. The possible use of lower flow rates, on the other hand, allowed the combination of a capLC to the mass spectrometer, which under normal conditions is not possible with commercial APPI sources, since these usually require flow rates in the order of 200 μL min^{-1} for appropriate ionization. The use of the heated nebulizer microchip at lower flow rates also produces a narrower gas jet, which provides high ion transfer into the mass spectrometer and therefore enhances the sensitivity of the method.

The positioning of the heated nebulizer microchip, the nebulizing gas (N_2) flow rate, the dopant (toluene) flow rate, and the heating power of the microchip significantly affected the signal intensity and stability. Therefore, careful positioning of the microchip and optimization of the aforementioned parameters were necessary. The APPI mass spectra of the analytes (17MDN, MTS, NAN and NANm) were acquired and the protonated molecule ($[M + H]^+$) of each analyte was chosen to be the precursor ion in the following SRM analyses. The collision energy (CE) was separately optimized for each analyte and MS/MS spectra were acquired. The most abundant and selective product ions were chosen for the SRM analyses.

The product ion chromatograms of the analytes in the spiked urine samples showed good chromatographic behaviour (Figure 8). All compounds were separated to baseline with repeatabilities of the retention times (%RSD t_R) below 0.9% (Table 11). The peak half-widths (W_h) and asymmetry factors (A_s) were 0.09-0.12 min and 1.04-1.67, respectively (Table 11). These results

indicate that the possible dead volume of the vaporizer channel in the microchip causes no peak broadening and that the microchip is suitable for this type of analyses. The lack of peak broadening also shows, that the temperature of the microchip was high enough to avoid adsorption of the analytes onto the surface of the microchip channel. Analyses of blank urine samples showed, that the chemical and biological background caused no interference in the analysis and no memory effect was observed after the analysis of the spiked urine sample (Figure 8).

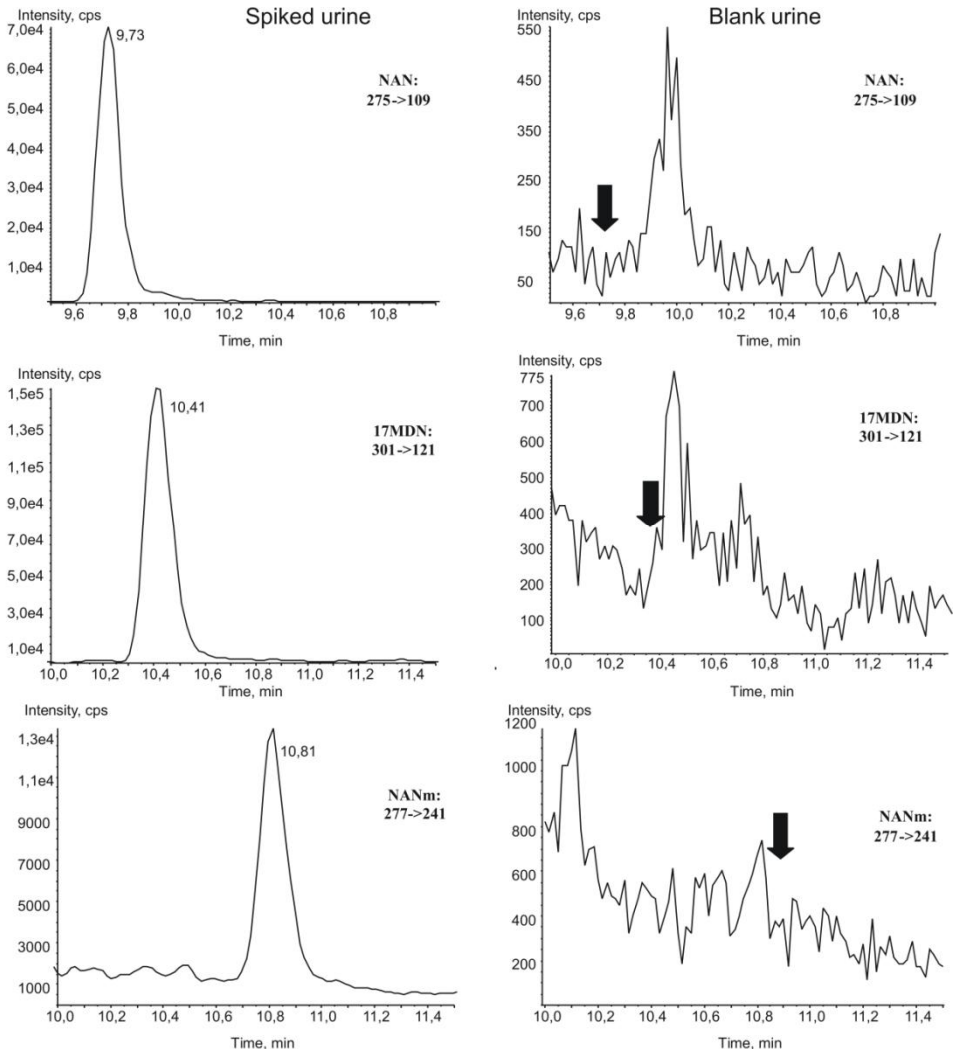


Figure 8 SRM chromatograms of the analytes obtained from spiked urine (50 ng mL^{-1}) and blank urine samples.

Table 11. *Retention times (t_R), repeatability of retention times (%RSD), peak widths at half-height (W_h), peak base width (W_b), and peak asymmetry (A_s). The concentration of the injected sample was 250 ng mL^{-1}, and the injection volume was 3 µL.*

Analyte	t_R (min)	%RSD t_R (N = 6)	W_h (min)	W_b (min)	A_s
17MDN	10.62	0.4	0.111	0.323	1.04
MTS	10.58	0.4	0.115	0.340	1.67
NAN	9.87	0.9	0.092	0.221	1.07
NANm	11.01	0.3	0.101	0.255	1.40

The developed method was evaluated with respect to linearity, limit of detection (LOD) and repeatability of injection. Full validation of the quantitative performance of this method was not carried out, because quantitative analysis is seldom necessary in doping analysis. In order to determine the linearities and LODs, the urine samples were spiked with the selected analytes to concentrations of 0.1, 0.5, 1, 2.5, 5, 7.5, 10, 25, 50, 100, 250 and 500 ng mL^{-1}, and pretreated as described in section 3.2.1. Calibration curves were obtained from the analyte to internal standard (MTS) peak area ratios by using linear regression with 1/x weighting. The coefficients of determination (R^2) were between 0.995 and 0.999, and the method was linear up to 500 ng mL^{-1} (17MDN and NANm) or up to 250 ng mL^{-1} (NAN) thus indicating adequate linearity of the method (Table 12). The LODs (at S/N = 3) were below 15 ng mL^{-1} for all of the compounds, which indicated reasonable sensitivity of the method. The reasonable sensitivity can be stated since the general minimum required performance level by the world anti-doping agency for exogenous anabolic androgenic steroids is 5 ng mL^{-1} and here two out of three of the measured compounds show LODs below this limit.

One urine sample was spiked to a concentration of 250 ng mL^{-1}, pretreated as described in section 3.2.1, and analyzed as six replicates in order to evaluate the repeatability of injection. The %RSDs of the peak areas were below 10% for all of the compounds, which indicated good repeatability of the method.

The results showed good quantitative behaviour of the developed method and that the method could be applicable for doping analysis, where quantitative analysis is seldom required. Nevertheless, more precise validation regarding the repeatability of the method at lower concentrations and optimization of the total analysis time should be conducted before implementation of the method in a routine laboratory.

Table 12. *Linear ranges, coefficients of determination (R^2), limits of detection (LODs), and repeatability of injection (%RSD).*

Analyte	R^2 (no. of points, range (ng mL^{-1}))	LOD (ng mL^{-1})	%RSD
17MDN	0.9990	0.5	9.3
	(10, 1-500)		
NAN	0.9954	0.3	4.2
	(9, 1-250)		
NANm	0.9964	15	8.8
	(5, 25-500)		

4.2 COMPARISON BETWEEN THE HPLC-CHIP/ESI/MS AND CONVENTIONAL LC/ESI/MS, AND ANALYSIS OF STEROIDS OF PLASMA SAMPLES BY THE HPLC-CHIP/ESI/MS

There are only a couple of commercial microchip based LC-MS systems available today. The feasibility of a commercial microchip based HPLC-Chip/MS system in the analysis of steroids was studied as one part of this study. The HPLC-Chip integrates a sample-enrichment column, a reversed phase LC separation column, and a nanoESI on a single polyimide chip. This system was used for the analysis of eight non-derivatized steroids in mouse plasma samples and the system was also compared to a conventional narrow-bore LC-MS system using oxime-derivatized steroids.

4.2.1 A COMPARISON BETWEEN THE HPLC-CHIP/MS SYSTEM AND THE CONVENTIONAL LC-ESI/MS SYSTEM

Oxime derivatives of ketosteroids were used in the comparison of the standard LC-MS with the HPLC-Chip/MS system. Oxime derivatization was chosen in order to enhance the ionization efficiency of the non-polar steroids in ESI and thus to enhance the sensitivity of the newly developed method. The ionization parameters were optimized for each analyte separately and MS spectra were acquired. All compounds showed abundant protonated molecules. These were chosen to be the precursor ions for the following SRM experiments, and MS/MS spectra were acquired. Product ions for the SRM experiments were chosen from the MS/MS spectra based on maximal sensitivity and selectivity and compound-specific SRM parameters were optimized.

The chromatographic performance of the conventional LC-ESI/MS was clearly better than the HPLC-Chip/MS system (Figure 9). The peaks were narrower (W_b = 0.22-0.38 min compared to W_b = 0.57-0.84 min) and the resolution was better for the LC-ESI/MS. The reason for this was probably

due to differences in column properties. The particle size in the conventional column was much smaller (1.8 µm) than in the chip column (5 µm). The pore size in the conventional column was also much smaller (80 Å against 300 Å in the chip column). Even though the resolution was poorer with the HPLC-Chip than with the conventional column, the co-eluting compounds could be separated using MS/MS. In many cases, however, isomeric steroids can not be separated by MS/MS and the separation relies on the chromatographic resolution. In these cases, the performance of the HPLC-Chip/MS system with 5 µm particles may not be sufficient to separate isomeric steroids in authentic biological samples. Another drawback of the HPLC-Chip is its narrow column (50 µm × 75 µm), since this limits the mass loading capability of the chip. The mass loading capability of the HPLC-Chip was tested by injecting 1.0 µL and 0.5 µL of a 15 nM standard sample. The results show, that an injection volume of 1.0 µL causes severe peak broadening compared to an injection volume of 0.5 µL (Figure 9A and B). With the conventional LC-ESI/MS, on the other hand, an injection volume of 40 µL could be used without causing peak broadening (Figure 9C).

Besides the chromatographic performance, the quantitative performances of the two systems were also compared. LODs, linearities and repeatabilities were evaluated using standard samples (Table 13). The LODs, when presented as concentrations, were found to be slightly higher for the HPLC-Chip/MS system (50-300 pM) than for the conventional LC-ESI/MS system (10-150 pM). However, when the LODs are expressed as injected amounts, the sensitivity of the HPLC-Chip/MS system, which used an injection volume of 0.5 µL was approximately 50 times higher compared to the conventional LC-ESI/MS system, which used an injection volume of 40 µL. The result is in agreement with the assumption that ESI/MS behaves more like a concentration-sensitive detector than a mass-flow sensitive detector, even if the mass spectrometer generally is a mass-flow sensitive detector. This can be explained by the fact, that the expected decrease in signal intensity due to decreased mass flow at lower flow rates is compensated by more efficient ion production.[177] This is because the initial charged droplet diameter decreases[178] and droplet charging becomes more efficient[179] with decreasing flow rates. This in turn leads to more efficient droplet disintegration and therefore to more efficient ion production at low flow rates.[180] The practical consequence is that the use of the HPLC-Chip/MS system is clearly advantageous in the analysis of low-volume samples.

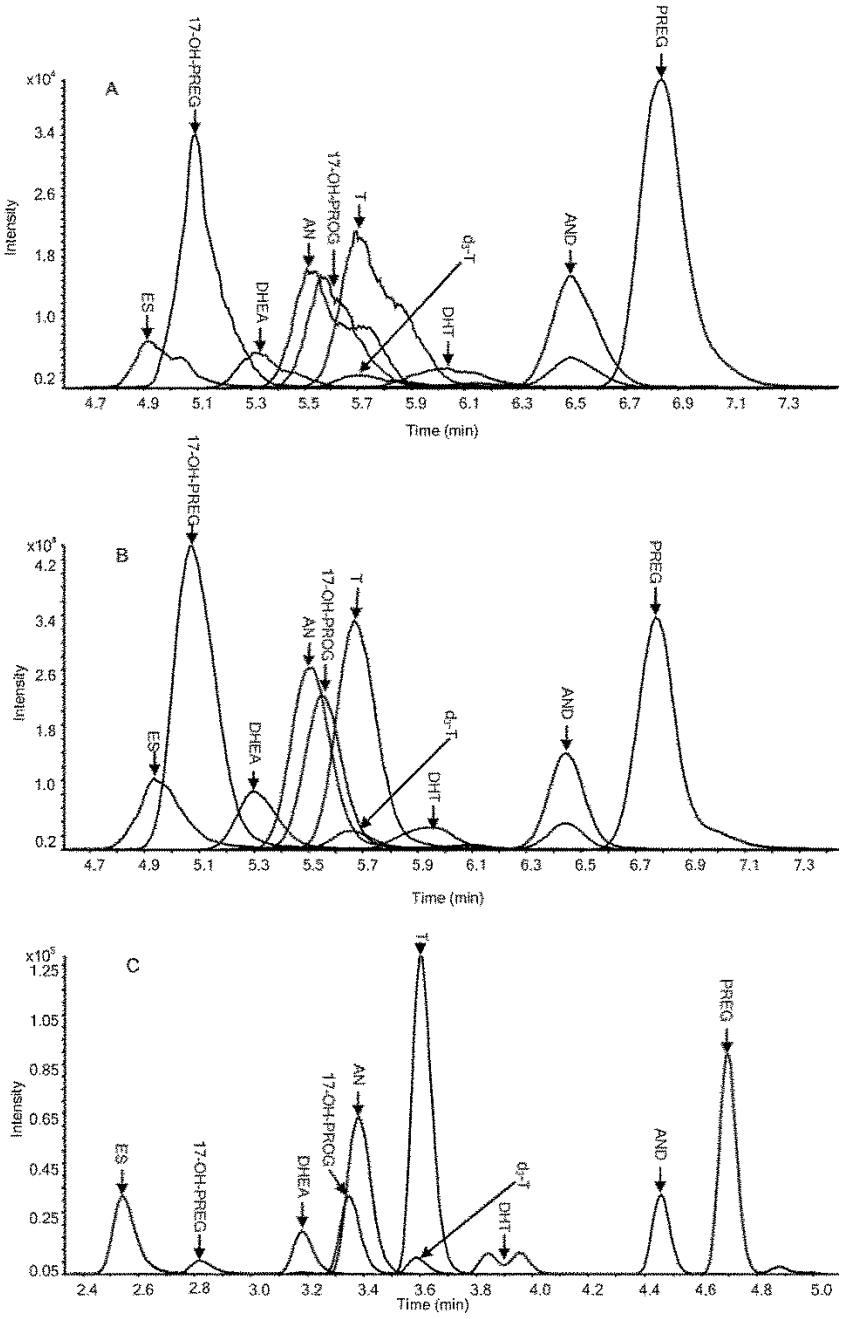


Figure 9 SRM chromatograms of the oxime derivatized steroids in standard samples (15 nM). (A) Injection volume of 1 µL with the HPLC-Chip/MS system, (B) injection volume of 0.5 µL with the HPLC-Chip/MS system and (C) injection volume of 40 µL with the conventional LC-ESI/MS system.

Table 13. *Limits of detection (LODs), coefficients of determination (R^2), repeatability of retention times (%RSD t_R), repeatability of peak areas (%RSD A), and carry-over of the HPLC-Chip/MS system for the oxime-derivatized steroids. The sample volumes injected were 40 µL in the conventional LC-ESI/MS system and 0.5 µL in the HPLC-Chip/MS system.*

Analyte	LOD pM (pg mL^{-1}), S/N > 3		R^2, range 0.075-180 nM		%RSD t_R (N = 5)		%RSD A (N = 5)		Carry-over of HPLC-Chip/MS system (%)
	LC-ESI/MS	HPLC-Chip/MS	LC-ESI/MS	HPLC-Chip/MS	LC-ESI/MS	HPLC-Chip/MS	LC-ESI/MS	HPLC-Chip/MS	
AN	30 (9)	50 (17)	0.9934	0.9993	0.5	0.1	6.1	2.7	4.1
AND	50 (15)	100 (29)	0.9970	0.9953	0.1	0.0	10.5	10.8	3.3
DHEA	75 (21)	100 (29)	0.9967	0.9979	0.5	0.1	9.1	2.4	3.5
DHT	150 (44)	300 (87)	0.9987	0.9975	0.4	0.2	6.8	3.5	5.2
ES	75 (20)	100 (27)	0.9969	0.9961	1.0	0.3	5.9	7.9	3.4
PREG	30 (10)	50 (16)	0.9944	0.9946	0.1	0.1	8.6	7.3	14.2
17-OH-PREG	75 (25)	100 (33)	0.9975	0.9981	0.7	0.0	7.0	5.9	3.4
17-OH-PROG	75 (25)	75 (25)	0.9956	0.9990	0.5	0.1	6.9	4.2	2.9
T	10 (3)	50 (14)	0.9961	0.9990	0.3	0.1	5.3	1.8	3.8

The coefficients of determination (R^2) were calculated using linear regression with 1/x weighting (Table 13). The results (0.993-0.999) were determined within a concentration range of 0.075-180 nM using 7-11 data points depending on the analyte. It can be seen, that based on the results both methods are linear within the application range and that they do not markedly differ from each other. The injection repeatability was also studied and compared between both systems. Five replicate injections were made using a 15 nM standard sample, and %RSDs were calculated for the retention times and for the peak areas (Table 13). The results show that %RSDs are below 1.0% for the retention times and below 11% for the peak areas. Additionally, the accuracies were well within the general range of 80-120%. These results indicate good chromatographic and quantitative repeatabilities and good accuracies of the methods.

Carry-over of the HPLC-Chip/MS system was tested in addition to the evaluation of the aforementioned general qualification parameters. This was done by injecting a reagent blank sample immediately after the analysis of a 15 nM standard sample. Peak areas from the standard and the blank samples were compared and a significant carry-over, typically between 2% and 5%, was detected (Table 13). This seemed to result from the built-in six-way microvalve of the microchip, and the problem was avoided by flushing the microvalve with a few reagent blank injections prior to the next sample injection. The conventional LC-ESI/MS, on the other hand, showed no carry-over.

4.2.2 THE FEASIBILITY OF THE HPLC-CHIP/MS SYSTEM FOR THE ANALYSIS OF NON-DERIVATIZED STEROIDS

Derivatization (e.g. oxime-derivatization) of steroids cannot be used for the development of a universal analysis method suitable for all steroids, since most of the existing derivatization procedures can only occur for steroids that possess a specific functional group.[64,117,181] The analysis of non-derivatized steroids, on the other hand, seldom provides the sensitivity needed for the analysis of actual biological samples. Nevertheless, the HPLC-Chip/MS system provides improved sensitivity when presented as injected amounts over the conventional LC-ESI/MS system and therefore the testing of this system with non-derivatized steroids was meaningful.

After optimizing the instrument parameters, MS and MS/MS spectra of the non-derivatized steroids were acquired. All steroids produced an abundant protonated molecule and a less abundant sodium adduct ion with minimal or no fragmentation. Product ions were chosen for SRM analyses in order to achieve maximum sensitivity and specificity and the compound specific SRM parameters were optimized.

The chromatographic performance of the HPLC-Chip/MS system for the non-derivatized steroids (Figure 10) was comparable to that for the derivatized steroids (Figure 9B). The peak widths (W_b) were 0.213-0.576 min, and no significant peak tailing was observed. The resolution was again insuffi-

cient to separate all the steroids, but the co-eluting compounds could be separated by MS/MS. These results, and the fact that no significant peak tailing can be observed, indicate reasonable chromatographic performance of the method with respect to the separation of the analytes.

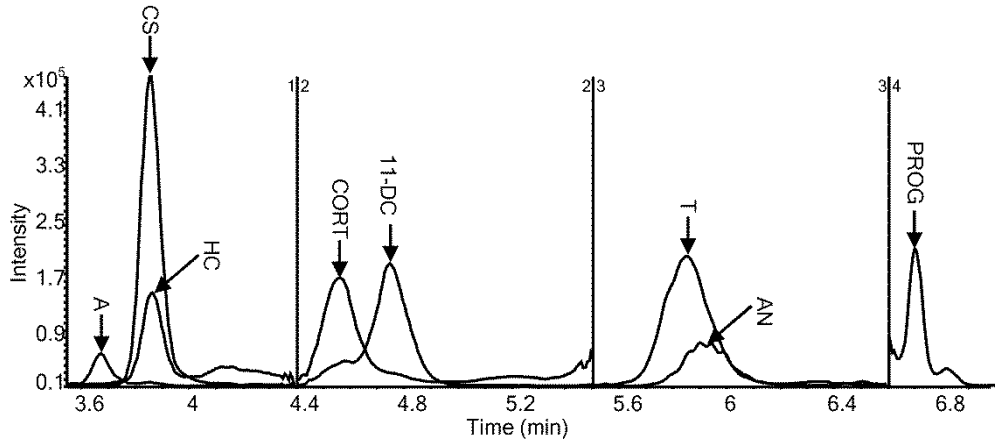


Figure 10 SRM chromatograms of the non-derivatized steroids in standard samples (7.5 ng mL^{-1}) using the HPLC-Chip/MS system. The injection volume was 0.75 µL.

The quantitative performance of the HPLC-Chip/MS system for the non-derivatized steroids was evaluated with respect to LODs, linearity, and repeatability using a spiked BSA-based artificial plasma matrix (Table 14). The LODs (S/N ≥ 3) ranged from 0.3 nM to 2.2 nM (0.1-0.8 ng mL^{-1}), which were about five to seven times higher than those determined for the oxime-derivatized steroids (Table 13). The higher sensitivity obtained for the derivatized steroids originates from the basic character of the oxime group, which significantly improves the ionization efficiency in ESI.[182] Calibration curves were measured at a concentration range of 0.075-100 ng mL^{-1} using 6-12 data points, depending on the analyte. The coefficients of determination (R^2) using linear regression with 1/x weighting were typically better than 0.990, which indicates acceptable linearity of a method developed based on new technology. The repeatability of injection was evaluated by injecting six replicates of a 25 ng mL^{-1} standard sample. The %RSDs were found to be below 0.5% and below 10% for the retention times and the peak areas, respectively. This indicates good repeatability of the method. The accuracies were within the general range of 80-120% indicating good accuracy of the method.

Table 14. *Limits of detection (LODs), coefficients of determination (R^2), repeatability of retention times (%RSD t_R) and repeatability of peak areas (%RSD A) for the non-derivatized steroids with the HPLC-Chip/MS system. The sample volume injected was 0.75 µL.*

Analyte	LOD nM (ng mL^{-1}) S/N ≥ 3	R^2, range 0.075-100 ng mL^{-1}	%RSD t_R (N = 6)	%RSD A (N = 6)
A	2.2 (0.8)	0.9878	0.5	9.2
AN	1.8 (0.5)	0.9964	0.3	4.6
CORT	0.7 (0.2)	0.9943	0.3	5.7
CS	0.3 (0.1)	0.9911	0.3	5.0
11-DC	0.3 (0.1)	0.9846	0.3	5.0
HC	0.7 (0.3)	0.9900	0.2	3.2
PROG	1.6 (0.5)	0.9750	0.1	6.5
T	1.7 (0.5)	0.9921	0.2	3.0

The feasibility of the HPLC-Chip/MS system was demonstrated in the analysis of non-derivatized steroids in eight female and seven male mouse plasma samples (Table 15). Of the eight steroids measured, only CORT could be detected in all of the samples at concentrations that ranged from 7.3 to 259 nM. The CORT concentrations in females were approximately six-fold (168 ± 64.1 nM) that of males (34.5 ± 11.1 nM). This result is in line with recent research results.[183] Various androgens were detected in some of the samples taken from males, but not in any of the samples from females. This difference results from the high variation in androgen concentrations in mouse plasma, as well as from their low concentrations in females. The rest of the analyzed steroids could not be detected in any of the samples.

In conclusion, the developed methods show acceptable quantitative behaviour for both oxime derivatized and non-derivatized steroids. The HPLC-Chip/MS system appears to be advantageous for low volume samples and it is feasible for the analysis of the selected non-derivatized steroids in mouse plasma samples. The conventional LC-MS system, on the other hand, showed slightly better sensitivities when expressed as the concentrations of the analytes present in the samples. Nevertheless, the methods presented here would need further validation and optimization in order to be able to achieve the often required pM or pg mL^{-1} sensitivity levels for steroid analysis. The results shown here suggest that this could possibly be achieved by using derivatization and the most sensitive mass spectrometer combined with the HPLC-Chip. Unfortunately this would limit the method to steroids having a specific functional group in their structure.

Table 15. *Quantified amounts of steroids present in mouse plasma samples. LLOQ stands for "lower limit of detection" and it means that the analyte was detected but due to its low amount it could not be quantified.*

Sample	Steroid, nM (ng mL^{-1})			
	CORT	A	T	AN
Females				
1	259 (90)	< LLOQ	-	-
2	75 (26)	< LLOQ	-	-
3	73 (25)	< LLOQ	-	-
4	182 (63)	< LLOQ	-	-
5	122 (42)	< LLOQ	-	-
6	124 (43)	< LLOQ	-	-
7	147 (51)	< LLOQ	-	-
8	77 (27)	< LLOQ	-	-
Males				
1	37 (13)	7.0 (2.5)	< LLOQ	-
2	7.3 (2.5)	3.7 (1.3)	24 (6.9)	3.2 (0.9)
3	17 (5.9)	-	-	-
4	23 (8.0)	-	< LLOQ	-
5	11 (3.8)	< LLOQ	< LLOQ	2.2 (0.6)
6	29 (10)	-	61 (18)	-
7	32 (11)	-	-	-

4.3 SEPARATION OF STEROID ISOMERS BY ION MOBILITY MASS SPECTROMETRY

Analysis of steroid isomers by conventional GC-MS and LC-MS techniques is generally considered time consuming and laborious, since it often requires very long chromatographic runs in order to achieve adequate separation of the isomers. Therefore, the feasibility of ESI-TWIM-MS in the analysis of selected non-derivatized and PTSI-derivatized steroid isomers was investigated. Additionally, changes in the separation efficiency of the steroid isomers as a result of using two different drift gases (N_2 and CO_2) at different pressures was studied.

Of the non-derivatized steroids, 17α-T and 17β-T were the best ionized in positive ion mode as $[M + H]^+$, whereas $[M + Na]^+$ dominated the spectra for 3α-AN and 3β-AN. The negative ion mode was more efficient for α-ES, β-ES and all the PTSI derivatives, thus these analytes were monitored as $[M - H]^-$.

Under optimized conditions the three different steroids (ES, AN and T) were successfully separated within milli-seconds by TWIM-MS (Figure 11). The α/β steroid isomers, however, could not be separated from each other

under any of the applied drift gas conditions. This is probably due to very similar collision cross sections of the isomers and the similar strengths of their ion/molecule interactions with the drift gas. This result was slightly unexpected, since it was assumed, that the more bent ring structure of the β-isomers (of the aliphatic steroids) would cause a more compact structure and therefore display shorter drift times compared to the α-isomers with more planar ring structures.

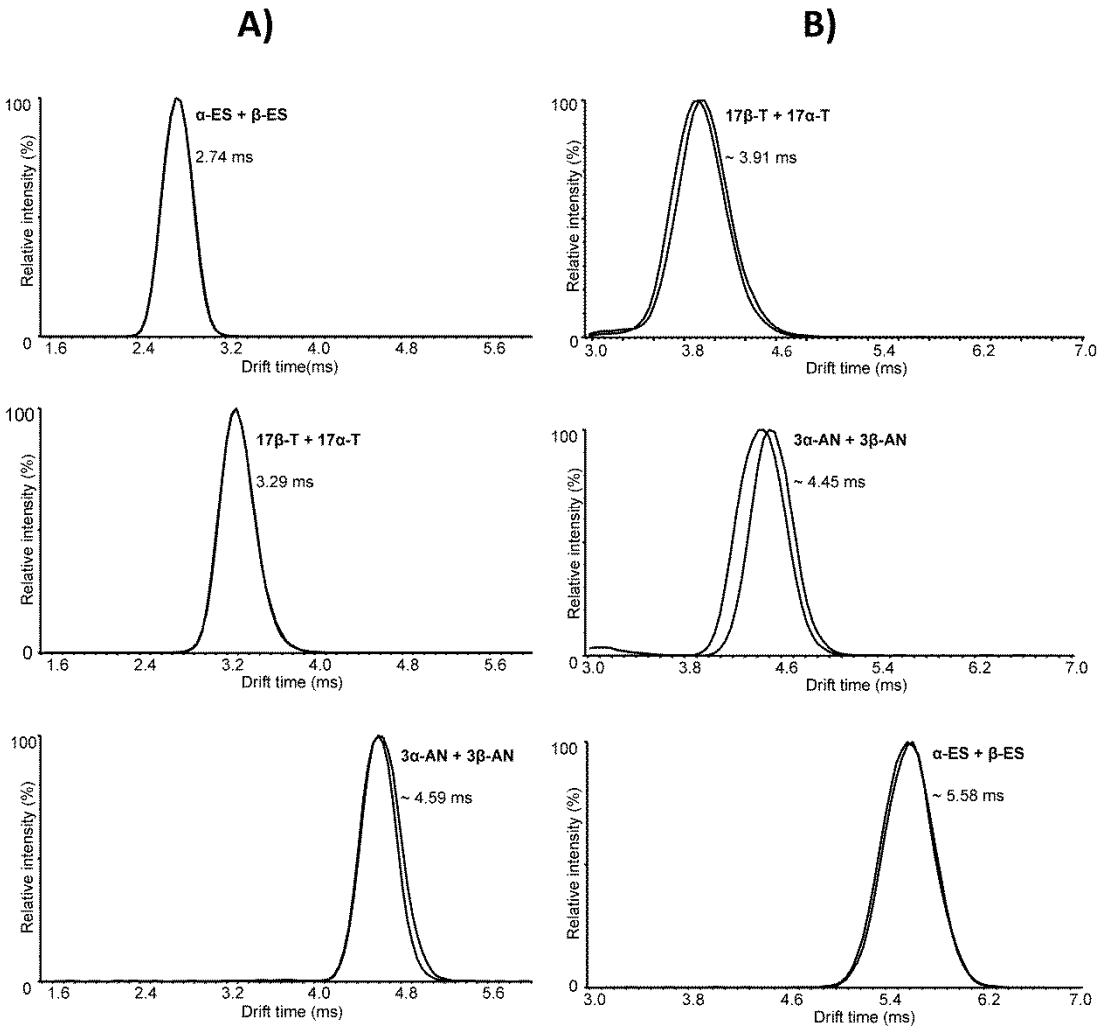


Figure 11 Selected ion mobility responses of the native steroid isomers (10 µg mL^{-1}): (A) N_2 (2.0 mbar) as the drift gas and (B) CO_2 (1.3 mbar for α-ES & β-ES and 17β-T & 17α-T, and 1.0 mbar for 3α-AN & 3β-AN) as the drift gas.

The steroid isomers could not be separated in their native forms, thus they were PTSI-derivatized in order to increase the difference of either the strength of their ion/molecule interactions with the drift gas and/or the difference in shape (i.e. their collision cross section). The derivatization facilitated sufficient separation efficiency for the steroid isomers. The TWIM-MS conditions and the ionization parameters were optimized for each PTSI derivatized isomer pair and the separation efficiency was evaluated in terms of peak-to-peak resolution ($R_{p\text{-}p}$) under different conditions (Table 16).

Table 16. *Peak-to-peak resolution of the TWIM-MS separation of the PTSI-derivatized steroid isomers.*

Isomer pair	CO_2 (mbar)		N_2 (mbar)		
	1.0	1.3	2.0	2.5	3.0
α-ES & **β**-ES	0	0.55	0.68	0.77	0.78
3α-AN & 3**β**-AN	0.90	0.98	0.92	1.08	1.24
17**α**-T & 17**β**-T	0.60	0.61	0.74	0.93	0.84

In addition to separation efficiency, the effects of different drift gas conditions on the sensitivity of the analysis were studied. When the pressure of N_2 was raised from 2.0 mbar to 3.0 mbar, and that of CO_2 raised from 1.0 mbar to 1.3 mbar, the $R_{p\text{-}p}$ between the different steroid isomers increased from 0.68-0.92 to 0.78-1.24 and from 0.00-0.90 to 0.55-0.98, respectively (Table 16). This indicates that $R_{p\text{-}p}$ increases as the drift gas pressure is raised. The sensitivity, however, decreased to about one-tenth when the pressure of N_2 was raised from 2.0 mbar to 3.0 mbar and between a half and one-third when the pressure of CO_2 was raised from 1.0 mbar to 1.3 mbar. The best compromise between $R_{p\text{-}p}$ and sensitivity based on these results was achieved at pressures of 2.5 mbar of N_2 or 1.3 mbar CO_2.

The comparison between drift gases at optimized pressures show that all three pairs of α/β isomers could be properly resolved (Figure 12). In other words, the $R_{p\text{-}p}$ values when using N_2 as the drift gas at a pressure of 2.5 mbar were 1.08, 0.93, and 0.77 for 3α-AN and 3β-AN, 17β-T and 17α-T, and α-ES and β-ES, respectively (Table 16). The corresponding values when using CO_2 as the drift gas at 1.0 mbar were 0.98, 0.61 and 0.55 (Table 16). These results, along with the $R_{p\text{-}p}$ values measured at other pressures (Table 16), indicate that using N_2 as the drift gas provides better separation efficiency compared to CO_2 for the PTSI-derivatized steroid isomers.

The performance of the analysis method was evaluated using N_2 as the drift gas at the pressure of 2.5 mbar. LODs and repeatabilities of injection and of the derivatization reaction were determined and are presented in Table 17. The calculated LODs (at S/N = 3) were below 25 ng mL^{-1} for all isomers, thus indicating reasonable sensitivity of the method. Repeatability of

injection was determined by five replicate injections of a standard sample at a concentration of 100 ng mL^{-1}. The results show good quantitative repeatability for all steroid isomers as the %RSDs were below 13%. Repeatability of the derivatization reaction was measured by injecting four separately prepared samples of the steroid isomers at a concentration of 10 µg mL^{-1}. The results show that the derivatization reaction is repeatable for all steroid isomers, with %RSDs below 7%. Since the drift times varied less than 2% between different measurements (N = 5) it could also be concluded, that the TWIM-MS method is very robust.

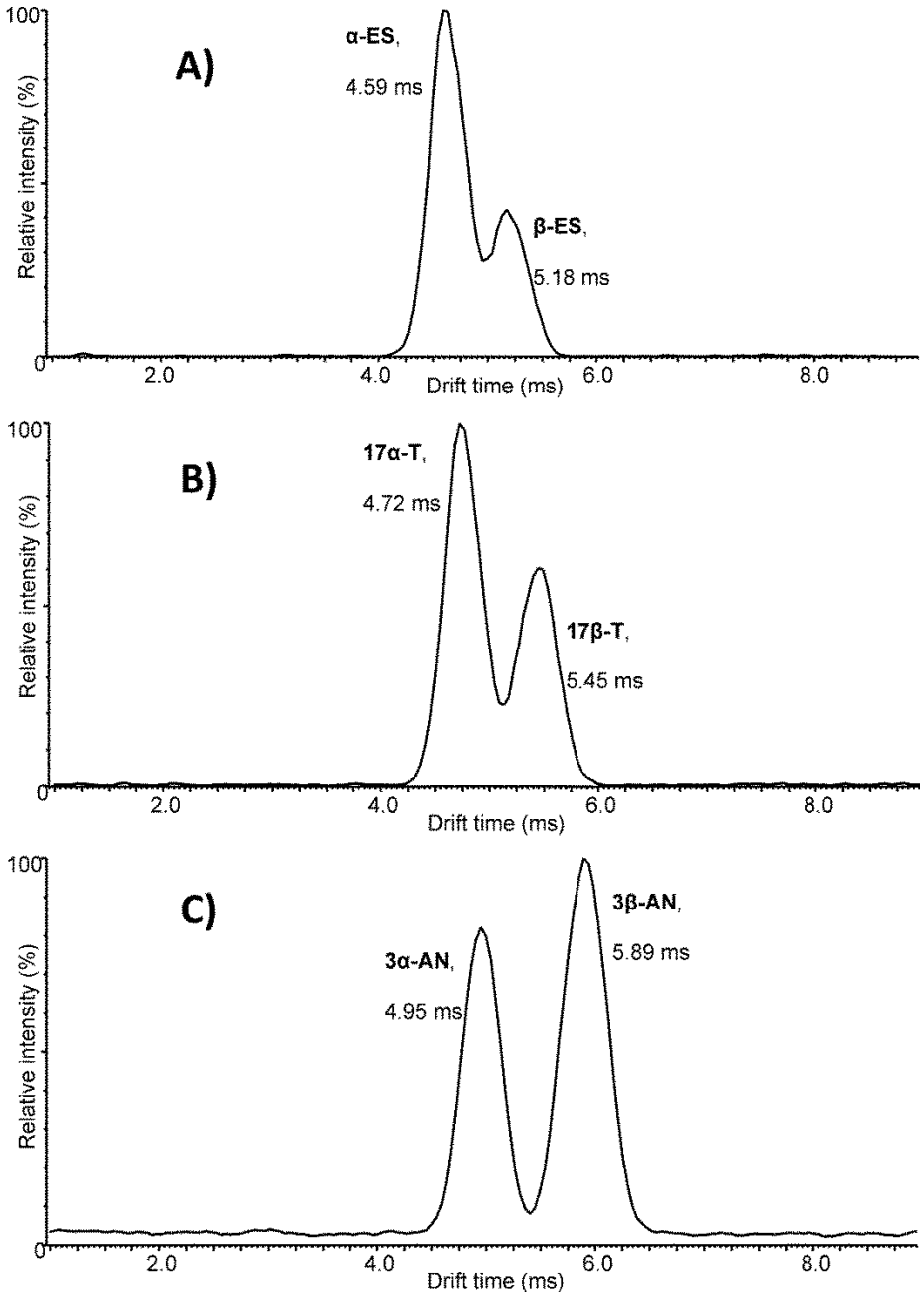


Figure 12 Selected ion mobility responses of the PTSI-derivatized steroid isomers (10 µg mL^{-1}) using N_2 (2.5 mbar) as the drift gas: (A) α-ES & β-ES, (B) 17β-T & 17α-T, and (C) 3α-AN & 3β-AN.

Table 17. *Limits of detection (LODs), repeatability of injection (%RSD A (injection)), repeatability of drift times (%RSD$_{drift-time}$ (injection)), and repeatability of the derivatization reaction (%RSD A (derivatization)) for the PTSI-derivatized steroid isomers.*

Steroid isomer	LOD (ng mL^{-1})	%RSD A (injection)	%RSD$_{drift-time}$ (injection)	%RSD A (derivatization)
		100 ng mL^{-1} (N = 5)		10 µg mL^{-1} (N = 4)
α-ES	4	5.1	0.9	1.9
β-ES	6	6.8	0.5	6.4
3α-AN	5	12.2	1.2	3.6
3β-AN	18	4.3	1.8	3.5
17**β**-T	15	7.3	0.8	4.3
17**α**-T	22	5.8	0.8	4.8

4.4 UHPLC-APPI-MS IN THE ANALYSIS OF VITAMIN D AND OXYSTEROLS OF BRAIN AND CELL LINE SAMPLES

Two UHPLC-APPI-based methods were developed for the simultaneous analysis of several oxysterols and vitamin D derived compounds in biological samples. First, the developed UHPLC-APPI-MS/MS method was successfully adapted to the analysis of mouse brain samples and to cell line samples. Non-stressed cell line samples were compared to cell line samples that had been exposed to oxidative stress and which were thus overexpressing alpha-synuclein (α-syn) aggregates (Parkinson's disease aggregates). Furthermore, the performance of the UHPLC-APPI-MS/MS method was compared to that of the other method (UHPLC-APPI-HRMS) in the analysis of cell line samples.

4.4.1 METHOD DEVELOPMENT AND VALIDATION

The APPI-MS spectra show abundant protonated molecules ($[M + H]^+$) and/or fragment ions that had been formed by the loss of one ($[M + H - H_2O]^+$), two ($[M + H - 2H_2O]^+$) or three ($[M + H - 3H_2O]^+$) water molecule(s). Based on the MS spectra, the most abundant precursor ions for the SRM experiments were chosen in order to maximize the sensitivity. MS/MS-spectra were acquired and product ions for the SRM experiments were chosen according to highest intensity and optimal selectivity.

The UHPLC method showed good chromatographic performance (Figure 13) with plate numbers and tailing factors within acceptable values (~ 40000 and < 1.5, respectively). Although the resolution was insufficient to achieve baseline separation of all the analytes, the co-eluting analytes could be separated by MS/MS. As an exception, the isomers 7α-OH-Chl and 7β-OH-Chl could not be separated from each other with sufficient resolution. Addition-

ally, the MS/MS spectra of these two compounds are similar, and therefore these isomers were quantified together.

The quantitative performance of the UHPLC-APPI-MS/MS system was evaluated with respect to LOD, linearity (R^2), intra-day repeatability and inter-day repeatability (Table 18). The LODs (at S/N ≥ 3) were below 2.5 ng mL^{-1} for all compounds except for 24-OH-Chl (LOD = 25 ng mL^{-1}), which indicated good sensitivity. Calibration curves and intra-day and inter-day repeatabilities were determined using analyte to internal standard (ISTD) peak-area ratios (the ISTDs were d_3-D_3 for D_2, D_3, DESMO and 7-DHYD, and d_6-27-OH-Chl for 1α,25-OH-D_3, 25-OH-D_3, 24-OH-Chl, 27-OH-Chl, 22-OH-Chl, 7α-OH-Chl, 7β-OH-Chl and 7-OXO). The calibration curves were determined within concentration range of 0.5-1000 ng mL^{-1} using at least six measuring points and linear regression with 1/x weighting. The coefficients of determination (R^2) were better than 0.998 for all of the compounds, which indicated that the method is linear over a broad range for all of the analytes. Seven replicate injections were carried out using a 100 ng mL^{-1} standard sample for the intra-day repeatability studies. The %RSDs were below 0.11% for the retention times, and below 5.89% for the analyte/ISTD peak area ratios. A 100 ng mL^{-1} standard sample was prepared on five consecutive days and seven replicate injections were made from each sample for the inter-day repeatability studies. The %RSDs were less than 0.68% for the retention times and less than 16.83% for the analyte/ISTD peak area ratios. The accuracies were well within the general range of 80-120%. These results indicate acceptable repeatability and accuracy of the method.

A UHPLC-APPI-HRMS method was used as a confirmatory method for the analysis of selected oxysterols and for the identification of other possible oxysterols in cell line samples. Therefore, the quantitative performance of the method was evaluated only with respect to the LODs and linearity (R^2) (Table 19). The LODs (at S/N ≥ 3) were 5 ng mL^{-1} for all of the compounds, which indicated that the UHPLC-APPI-MS/MS method provides slightly better sensitivity for most of the analytes compared to the corresponding values for the UHPLC-APPI-HRMS method. Calibration curves were determined using the analyte/ISTD (d_6-27-OH-Chl) peak area ratios and linear regression with 1/x weighting. The measuring range was 10-500 ng mL^{-1} (six measuring points) or 20-500 ng mL^{-1} (five measuring points) depending on the analyte. The coefficients of determination (R^2) exceeded 0.991 for all of the compounds and the accuracies were within the general range of 80-120%. These results indicate good linearity of the method. The accurate masses of the compounds were measured showing errors less than 3 ppm.

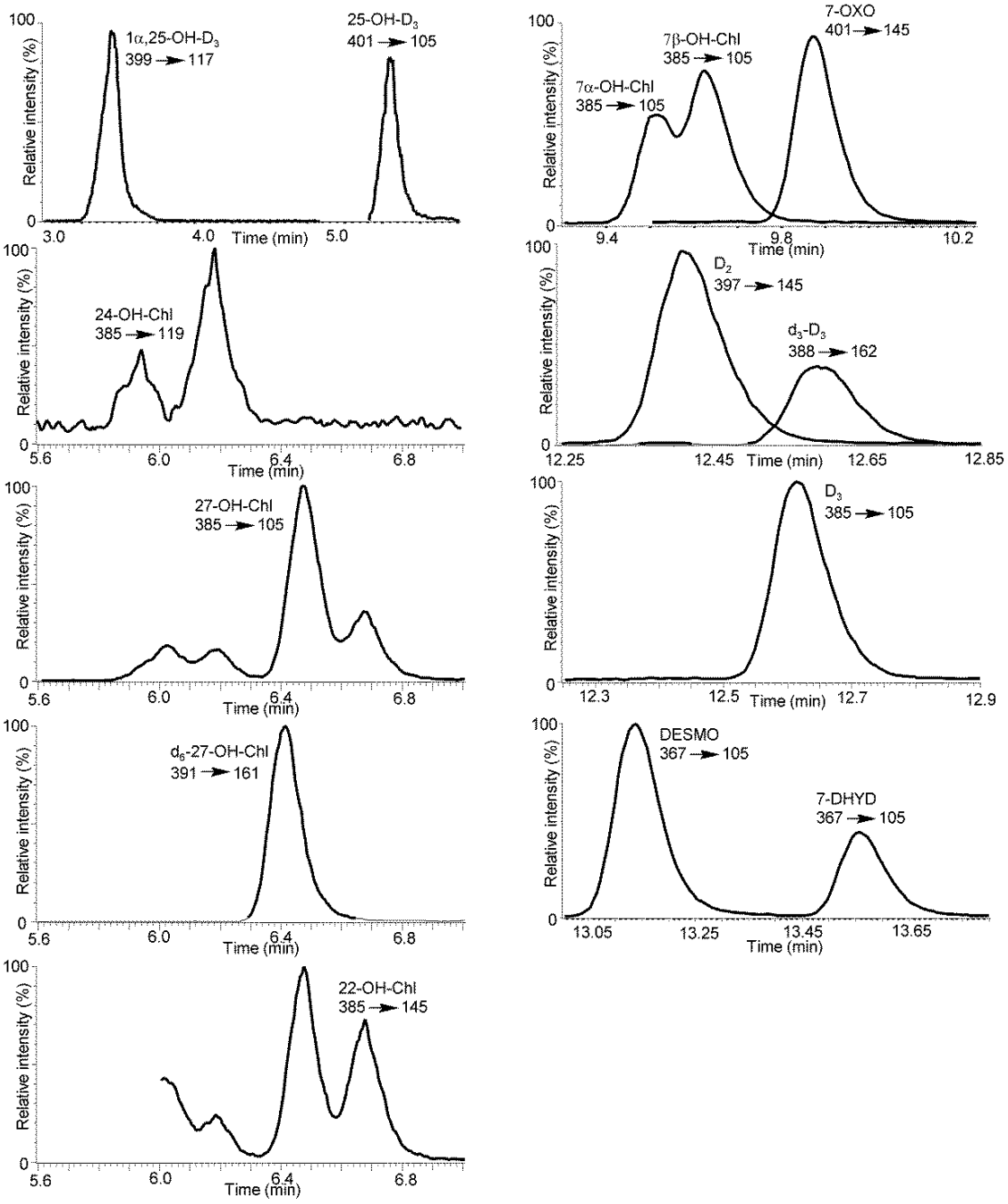


Figure 13 SRM chromatograms of the analytes in a 100 ng mL^{-1} standard sample analysed by UHPLC-APPI-MS/MS.

Table 18. *Limits of detection (LODs), limits of quantitation (LOQs, determined at S/N ≥ 10), coefficients of determination (R^2), retention times (t_R), intra- and inter-day repeatabilities of retention times (%RSD_{inj}_t_R and %RSD_{inter}_t_R) and intra- and inter-day repeatabilities of peak areas (%RSD_{inj}_A and %RSD_{inter}_A) for the analytes measured using the UHPLC-APPI-MS/MS method.*

Analyte	LOD/LOQ (ng mL^{-1})	R^2/range (ng mL^{-1})	t_R (min)	%RSD_{inj}_t_R (N = 7)	%RSD_{inj}_A (N = 7)	%RSD_{inter}_t_R (N = 35, 5 × 7)	%RSD_{inter}_A (N = 35, 5 × 7)
1α,25-OH-D_3	0.5/1.0	0.9992/0.5-1000	3.50	0.11	5.89	0.68	16.83
25-OH-D_3	2.5/2.5	0.9993/2.5-1000	5.38	0.09	2.87	0.64	15.89
24-OH-Chl	25/100	0.9989/25-500	6.07	0.08	6.16	0.58	5.73
27-OH-Chl	1.0/5.0	0.9990/ 1-1000	6.56	0.07	4.48	0.56	5.01
22-OH-Chl	2.5/5.0	0.9995/2.5-1000	6.76	0.00	3.38	0.58	4.87
7α-OH-Chl + **7β**-OH-Chl	2.5/2.5	0.9996/2.5-500	9.61 + 9.72	0.00	5.64	0.53 + 0.52	6.81
7-OXO	1.0/1.0	0.9996/1-500	9.97	0.00	3.47	0.51	11.74
D_2	1.0/2.5	0.9994/1-1000	12.52	0.00	3.21	0.46	4.03
D_3	1.0/2.5	0.9992/1-500	12.72	0.00	1.96	0.43	4.45
DESMO	1.0/2.5	0.9989/1-500	13.24	0.03	2.28	0.43	11.74
7-DHYD	1.0/5.0	0.9988/1-1000	13.65	0.03	2.67	0.45	4.78

Table 19. *Limits of detection (LODs), limits of quantitation (LOQs, determined as S/N ≥ 10), coefficients of determination (R^2), exact and accurate masses of the analytes measured using UHPLC-APPI-HRMS.*

Analyte (detected ion)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	R^2 range (ng mL^{-1})	Formula	Exact mass	Accurate mass	Error (ppm)
24-OH-Chl ([M+H-2H$_2$O]$^+$)	5	20	0.9947 20-500	$C_{27}H_{43}$	367.3365	367.3367	0.54
27-OH-Chl ([M+H-H$_2$O]$^+$)	5	10	0.9959 10-500	$C_{27}H_{45}O$	385.3470	385.3477	1.82
7α-OH-Chl ([M+H-2H$_2$O]$^+$)	5	20	0.9962 20-500	$C_{27}H_{43}$	367.3365	367.3367	0.54
7β-OH-Chl ([M+H-2H$_2$O]$^+$)	5	20	0.9955 20-500	$C_{27}H_{43}$	367.3365	367.3367	0.54
7-OXO ([M+H]$^+$)	5	20	0.9966 20-500	$C_{27}H_{45}O_2$	401.3420	401.3432	2.99
7-DHYD ([M+H-2H$_2$O]$^+$)	5	10	0.9907 10-500	$C_{27}H_{43}$	367.3365	367.3367	0.54
DESMO ([M+H-2H$_2$O]$^+$)	5	20	0.9953 20-500	$C_{27}H_{43}$	367.3365	367.3367	0.54

4.4.2 FEASIBILITY OF THE METHODS IN THE ANALYSIS OF BIOLOGICAL SAMPLES

The feasibility of the UHPLC-APPI-MS/MS method for the analysis of vitamin D related compounds and oxysterols in biological samples was demonstrated by analyzing six mouse brain samples and eight human neuroblastoma cell line samples. Non-stressed cell line samples were compared to cell line samples that had been exposed to oxidative stress and thus were overexpressing alpha-synucelin (α-syn) aggregates (Parkinson's disease aggregates). The analytes were identified according to the following criteria: the variation of the intensity ratios of the monitored product ions was less than 15% and the variation of the retention times was less than 0.25% compared to the standard sample. The feasibility of the UHPLC-APPI-HRMS method, on the other hand, was demonstrated by analyzing oxysterols in non-stressed and stressed cell line samples. The analytes were identified using a 0.25% retention time window and a 25 mDa mass window.

Seven of the selected analytes were found in the mouse brain samples (Table 20). The most abundant oxysterol in all of the brain samples was 24-OH-Chl, which was detected at a high concentration (1.0-1.6 × 10^5 ng g^{-1}). This r5esult could be due to 24-OH-Chl being synthesized in the brain directly from cholesterol by CYP46 catalyzed reactions and only 20% of which is transported into the general blood flow across the blood-brain barrier.[79,184-186] DESMO and 27-OH-Chl were also detected in the brain samples at relatively high concentrations (8.7-12.3 × 103 ng g^{-1} and 90-210 ng g^{-1}, respectively), which agrees with other studies.[79,187-189] 27-OH-Chl is formed from cholesterol in an enzymatic reaction that is catalyzed by sterol 27-hydroxylase and it is further metabolized by the same enzyme to 3β-hydroxy-5-cholestenoic acid.[46,190,191] This cholestenoic acid is metabolized in a oxysterol 7α-hydroxylase catalyzed reaction to the metabolite 7α-hydroxy-3-oxo-4-cholestenoic acid, which is efficiently transferred from the brain to the circulation across the blood brain barrier. This could be the reason for the higher concentrations of 24-OH-Chl in the mouse brains compared to 27-OH-Chl.[188] 7α-OH-Chl/7β-OH-Chl (103-136 ng g^{-1}) and 7-OXO (~ 60 ng g^{-1}) were also detected in the mouse brain samples. These compounds have previously been identified in human brain samples.[189,192] One noteworthy analyte, namely 25-OH-D_3, was detected in all of the brain samples at low concentrations (~4 ng g^{-1}) using SRM transitions of *m/z* 401 → *m/z* 105 and *m/z* 133. The finding was also analyzed using, SRM transitions of *m/z* 383 ([M + H - H_2O]$^+$) → *m/z* 105 and *m/z* 133. The identification criteria were fulfilled in both analyses, thus the detection of 25-OH-D3 in mouse brain samples was confirmed for the first time with relatively high reliability although the ion chromatograms are quite noisy (Figure 14).

Table 20. *Concentrations of the analytes found in the mouse brain by UHPLC-APPI-MS/MS.*

Sample	Compound (ng g^{-1})					
	25-OH-D_3	24-OH-Chl (× 10^3)	27-OH-Chl	7α- & 7β-OH-Chl	7-OXO	DESMO (× 10^3)
1	3.40	1.00	89.7	136	75.9	8.71
2	3.44	1.20	130	104	53.8	12.3
3	3.11	1.19	152	108	59.3	9.33
4	3.84	1.21	145	108	56.7	9.54
5	3.76	1.59	209	106	56.3	10.0
6	5.22	1.40	204	132	62.4	7.95

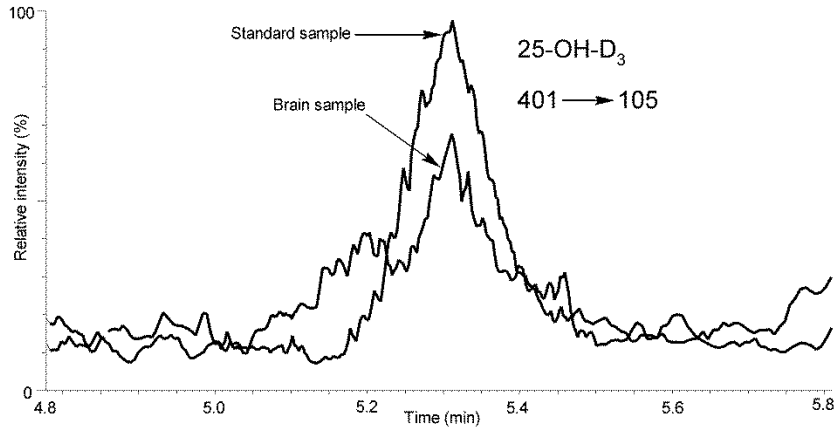


Figure 14 Overlaid SRM-chromatograms of the detected 25-OH-D_3 in a standard sample and in a mouse brain sample.

Four oxysterols (24-OH-Chl, 7α-/7β-OH-Chl, 7-OXO and DESMO) were identified in the cell line samples and their concentrations were in good agreement with previous studies (Table 21 and Table 22).[46] The concentrations of 7α-/7β-OH-Chl and 7-OXO were more than tenfold in the stressed cell line samples than those of the non-stressed samples. This indicates that these oxysterols are readily formed by the oxidation of cholesterol in the stressed cell line samples. DESMO was also detected in all of the cell line samples. Interestingly, the concentration of this compound is half to one-third in the stressed samples to that of the non-stressed samples. This may be due to further oxidation of the double bond at the side chain of this compound. Similar results have been observed by other research groups as well.[193,194] Additionally, 24-OH-Chl was detected in the non-stressed cell line samples at concentrations below LOQ when the UHPLC-APPI-MS/MS method was used. Therefore, a reliable quantification of this compound could not be achieved. On the other hand, 24-OH-Chl was not detected in the stressed cell line samples.

Table 21. *Concentrations of the analytes found in cell line samples by UHPLC-APPI-MS/MS.*

Sample	Compound (ng mL^{-1})			
	24-OH-Chl	7α- & 7β-OH-Chl	7-OXO	DESMO
WT 1	< LOQ	75.5	146	40
WT 2	< LOQ	29.3	39.7	27
WT 3	< LOQ	20.9	32.9	24
WT 4	< LOQ	24.9	42.7	28
WT 5	< LOQ	28.3	41.4	27
WT 6	< LOQ	26.6	43.1	28
Stressed WT 1	< LOQ	274	919	6.9
Stressed WT 2	< LOQ	316	1070	7.7
Stressed WT 3	< LOQ	326	1080	9.1
A30T 1	< LOQ	25.3	37.2	20
A30T 2	< LOQ	23.1	33.3	20
A30T 3	< LOQ	25.4	36.1	21
Stressed A30T 1	< LOQ	398	976	3.2
Stressed A30T 2	< LOQ	290	715	2.7
Stressed A30T 3	< LOQ	293	715	2.5

Table 22. *Concentrations of the analytes found in cell line samples by UHPLC-APPI-HRMS.*

Sample	Compound (ng mL^{-1})			
	7α-OH-Chl	7β-OH-Chl	7-OXO	DESMO
WT 1	10.8	41.5	103	16
WT 2	14.2	46.0	125	21
WT 3	21.6	57.1	142	19
Stressed WT 1	234	1000	1970	4.0
Stressed WT 2	187	813	1630	-
Stressed WT 3	301	1080	2120	5.9
A53T 1	22.9	44.2	105	14
A53T 2	81.8	111	266	20
A53T 3	20.8	38.7	96.7	18
Stressed A53T 1	286	794	1770	4.6
Stressed A53T 2	288	771	1760	-
Stressed A53T 3	263	731	1650	-

The data produced by UHPLC-APPI-HRMS can also be used in identifying non-targeted oxysterols and vitamin D related compounds. In this study four additional compounds, the concentrations of which were higher in the stressed cell line samples compared to the non-stressed cell line samples, were identified by the UHPLC-APPI-HRMS method. These four compounds were identified as oxysterols based on data that met the following criteria: the collision induced spectra (CE = 30 eV) of these compounds showed similar types of fragmentation as the selected oxysterols, the exact masses corresponded to the elemental composition of oxysterols, and the retention times were in the same range as the retention times for the selected oxysterols. The data did not, however, allow the characterization of the exact structures of these compounds.

In conclusion, two UHPLC-APPI-MS methods were developed and qualified for the simultaneous analyses of several oxysterols and vitamin D related compounds in the mouse brain and cell line samples. The concentrations of the detected oxysterols in the brain samples were consistent with earlier studies and additionally, 25-OH-D_3 was identified and quantified in mouse brain samples for the first time. The concentrations of two of the compounds (24-OH-Chl and DESMO) were over the linearity range of the calibration curve. Thus, the determined concentrations of these compounds are not exact, but for the purpose of this study the exact concentrations of these analytes were not considered relevant and therefore no further measurements (for example after diluting the samples) were made. Oxysterols were detected in the brain samples, therefore they were also analyzed in cell line samples

used as a model to study the formation of α-syn aggregates related to Parkinson's disease. Here oxidative stressed cell line samples that overexpress α-syn were compared to non-stressed samples. The concentrations of the oxysterols differed remarkably in the oxidative stressed cell line samples compared to the non-stressed controls. These results show that the role of oxysterols in the formation of α-syn aggregates cannot be ruled out and further studies are needed for determining and elucidating the roles of oxysterols in brain function.

5 SUMMARY AND CONCLUSIONS

The overall aim of this research was to develop different mass spectrometry based methods for the analysis of steroids, vitamin D related compounds and oxysterols, and to apply the developed methods in the analysis of biological samples.

It was shown (I), that the in-house-built heated-nebulizer microchip in APPI mode (μAPPI) combined to a capLC-MS/MS is feasible for the analysis of anabolic steroids in human urine samples. The quantitative performance of the developed method was evaluated with respect to the limits of detection, linearity and repeatability, and the results show good quantitative performance of the method. Full validation of the method was not carried out, since quantitative analysis is seldom necessary in doping analysis. The advantage of μAPPI over commercial APPI sources, is the possibility of using low flow rate capLC, which is not possible with commercial APPI sources since they require flow rates in as high as 200 μL min^{-1} for adequate ionization of the analytes. The advantage of using capLC, on the other hand, is that the ionization efficiency can be improved since the lower flow rate produces a very narrow gas jet, which provides high ion transmission into the mass spectrometer. Additionally the use of capLC results in decreased eluent, reagent, and nebulizing gas consumption thus decreasing analysis costs and environmental load. Therefore, it can be concluded that capLC-μAPPI-MS/MS is a promising tool for bioanalysis.

The feasibility of a commercial microfluidic-based HPLC-Chip/MS system for the analysis of oxime-derivatized steroids was compared to a conventional narrow-bore LC-MS system in study II. Both methods showed good linearity and good quantitative and chromatographic repeatability. It was also shown, that the sensitivity of the HPLC-Chip/MS system was about 50 times better that of the sensitivity of the conventional narrow-bore LC-MS system if the limits of detection are presented as injected sample amounts. The sensitivity of the conventional LC-MS system is, however, slightly better when the limits of detection are presented as concentrations in the sample. It follows that the use of the HPLC-Chip/MS system is advantageous when the sample volume is limited, which often is the case in the analysis of biological samples.

A comparison of the analysis of oxime-derivatized steroids using the HPLC-Chip/MS system with that of the analysis of non-derivatized steroids in study II, found that the sensitivity with the oxime derivatives were approximately one order of magnitude higher than those of the non-derivatized steroids. This result is in agreement with previous studies and it is because the basic character of the oxime group significantly improves the ionization efficiency in ESI. Nevertheless, the feasibility of the HPLC-Chip/MS system for the analysis of non-derivatized steroids in mouse plasma samples was

successfully demonstrated for the selected steroids and showed steroid concentrations within the same range as previously reported. This information is useful, since oxime derivatization can be applied only for ketosteroids and thus the development of a universal method for steroids by means of oxime derivatization is impossible. Nevertheless, the method should be further validated with respect to repeatability and regarding possible matrix effects. Now the matrix effect was only tested by injecting blank artificial plasma samples prior and after the spiked artificial plasma samples. Post-column infusion could unfortunately not be made with the HPLC-Chip/MS system since the chip is inserted in the Chip Cube and no additional connections can be made between the column and the mass spectrometer. Additionally, another type of HPLC-Chip should be chosen for future work, since the particle size (5 µM) and the pore size (300 Å) in the column used for this study were not small enough. This might solve the problem with wide peaks and co-elution of analytes and thus it would provide better selectivity of the system (especially regarding isomeric steroids).

The work presented in publication III demonstrates the feasibility of TWIM-MS for separating steroid isomers under different drift gas conditions. The final method used the derivatization of the steroid isomers with *p*-toluenesulfonyl isocyanate, since the collision cross sections of the native steroid isomers appeared to be too similar for successful separation by TWIM-MS. The derivatization increased the collision cross section of the isomers and/or the strength of their ion/molecule interactions with the drift gas and hence it improved their separation in the ion mobility cell. As a result, the steroid isomers could be successfully separated and showed almost baseline resolution. This is a very important result for steroid analysis (e.g. within doping analysis) since TWIM-MS can be performed on a millisecond time-scale. Therefore, this method can improve immediate separation, characterization and quantification of α/β steroid isomers. Unfortunately the separation of the steroid isomers requires the derivatization step, which increases the analysis time. Nevertheless, the derivatization step can be done in parallel with the analysis step and thus high throughput is still possible with the developed TWIM-MS method. The sensitivity of the developed method might cause problems for the analysis of some biological samples where the background signal caused by the matrix is high (for example urine samples). This problem could be solved, though, by combining the TWIM-MS method with a UHPLC-MS/MS method whenever additional high sensitivity is needed.

In publication IV, a UHPLC-APPI-MS/MS method was developed for the simultaneous quantitative analysis of several oxysterols and vitamin D related compounds in the mouse brain and cell line samples. The results achieved for the cell line samples were confirmed by a UHPLC-APPI-HRMS method. Seven oxysterols could be quantified from mouse brain samples by the UHPLC-APPI-MS/MS method. The results were consistent with earlier findings obtained by LC-ESI-MS methods. Interestingly, 25-OH-D_3 was also

identified and quantified for the first time in mouse brain samples. Additionally, both oxidative stressed and non-stressed cell line samples were analysed. Four of the selected compounds could be identified and quantified at differing concentrations, depending on whether the sample had been oxidative stressed or not, by the UHPLC-APPI-MS/MS and the UHPLC-APPI-HRMS method. Data acquired by the UHPLC-APPI-HRMS method also showed the presence of four non-targeted oxysterols in all of the cell line samples within the same concentration range as the targeted analytes. These results suggest, that the role of oxysterols in the formation of α-syn aggregates related to Parkinson's disease cannot be rule out. Consequently, further studies are needed for understanding the actual roles of oxysterols in brain function.

The advantage of using APPI in publication IV instead of the more common ESI is that the time consuming and laborious derivatization procedure usually required in ESI is not needed in APPI to achieve adequate ionization efficiency. Therefore, APPI provided significantly faster analyses for the selected compounds compared to ESI. Thus the developed UHPLC-APPI-MS methods are well suitable for further studies related to role of oxysterols in brain function.

In conclusion, the work summarized in this thesis presents several new methods for the analysis of selected lipids in biological samples. Routine laboratories e.g. within forensic and doping analysis all over the world constantly need new highly sensitive and selective methods. Additionally, the methods should be easy and fast, and they should have lower analyses costs and decreased environmental impact. Mass spectrometry based analysis of lipids is more problematic compared to the analysis of more polar analytes, therefore the importance of the development of new methods within the field of lipid analysis is highly important. Additionally, exact knowledge of the importance of lipids in biological systems such as the human brain, in which minimal changes in concentration levels might affect the function of the entire system would be a boon to neurological research. This is due to the fact that the concentrations of many of the studied compounds might be below the detection limits of the current analytical methods. Some of the methods presented here could be directly implemented in routine laboratories, while some would need additional optimization for example regarding validation of the methods, detection limits (especially the often required sensitivity of 1 pM for steroid analysis remain a challenge) and total analysis times. Especially the sensitivity issues should be addressed before implementing these methods in routine laboratories. Nevertheless, some of the presented methods (I and IV) show sensitivities and qualification parameters, which are well comparable to previously presented methods (Table 1) and thus applicable for routine analysis. All in all, the new information presented in this thesis can be important for routine laboratories in the future.

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APPENDIX: ORIGINAL PUBLICATIONS I-IV

Some minor mistakes in the original publications were found when writing this thesis. These mistakes have been corrected in the thesis, and are as follows:

- Original publication I, Table 4: the linear range and number of measuring points for NAN are wrongly written. The correct values are 1-250 ng mL^{-1} for the linear range (this is written correctly in the text on page 963) and 9 measuring points.

- Original publication I, Sections "Abstract" (page 958) and "Results and discussion" (page 963) and Table 4: the term "correlation coefficient" is wrongly used in combination with the abbreviation R^2. The correct term for this abbreviation is "coefficient of determination".

- Original publication II, Section 2.5 (page 117): eluent A and eluent B have been mixed up when explaining the gradient for non-derivatized steroids. The correct eluent are (A) 50 mM NH_4Ac (pH 4.5):H_2O (1:9, v/v) and (B) 50 mM NH_4Ac (pH 4.5):MeOH:ACN (1:3:6, v/v/v).

- Original publication II, Sections 3.1 (page 119) and 3.2 (page 119), and Tables 1 and 2: the term "correlation coefficient" is wrongly used in combination with the abbreviation R^2. The correct term for this abbreviation is "coefficient of determination".