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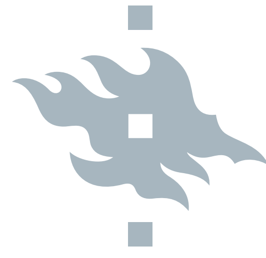
SIRKKU JÄNTTI Liquid Chromatography–Mass Spectrometry in Studies of Steroids and Steroid Glucuronides in Brain and Urine



DISSERTATIONES BIOCENTRI VIIKKI UNIVERSITATIS HELSINGIENSIS 19/2013

SIRKKU JÄNTTI

Liquid Chromatography-Tandem Mass Spectrometry in Studies of Steroid Hormones and Steroid Glucuronide Conjugates in Brain and Urine

DIVISION OF PHARMACEUTICAL CHEMISTRY
FACULTY OF PHARMACY
UNIVERSITY OF HELSINKI

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Liquid Chromatography-Tandem Mass Spectrometry in Studies of Steroid Hormones and Steroid Glucuronide Conjugates in Brain and Urine

Sirkku Jäntti

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in lecture room 2 at Viikki C-building (Latokartanonkaari 5) on October 4th, 2013, at 12 noon.

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Abstract

Steroids are endogenous compounds, which can be classified based on their biological activities into estrogens, androgens, progestogens, glucocorticoids and mineralocorticoids. Steroids are present in the body tissues and fluids in free and conjugated forms, for example conjugation with glucuronide acid, sulphate, fatty acids, and amino acids, or they can be bound to proteins. To study the steroids in biological samples, suitable analytical methods are required for their analysis. Traditional methods, such as radioimmuno assay (RIA) and gas chromatography-mass spectrometry (GC-MS), have many limitations. Cross reactions, selectivity and the use of radioactive labels restricts use of RIA. In RIA specific antibodies are needed for analysis, are not available for all steroid glucuronides. GC-MS rely on the detection of hydrolysed and derivatized steroid aglycons and for that laborious sample preparation must be carried out. Hydrolysis can also alter or decrease the information obtained from the analysis: the origin of a conjugate can be ambiguous, the information of the conjugation site(s) is lost, and incomplete hydrolysis is possible. In addition, impurities of enzyme preparates used in hydrolysis, for example β-glucuronidase and sulphatase enzyme, can lead unwanted side products. Using liquid chromatography - mass spectrometry (LC-MS) with soft ionisation methods, such as electrospray ionisation (ESI), steroid glucuronides can be analysed in their intact form.

In this study, one goal was to develop methodologies for the synthesis of steroid glucuronide conjugates. Reference materials were needed for development and validation of the methods, since the commercial availability of steroid glucuronides is limited. An enzyme-assisted synthesis was carried out to produce glucuronide-conjugated steroids at milligram levels. Hepatic microsomal preparations of bovine, porcine, and Arochlor-induced rats were compared with respect to specificity and efficiency of uridine diphosphate glucuronosyl transferase (UGT) enzymes in steroid glucuronidation. Both bovine and porcine liver microsomes efficiently produced the steroid glucuronides of all eight steroids studied, whereas rat liver microsomes produced glucuronides efficiently only for three steroids. Synthesized steroid glucuronides were purified with solid-phase extraction (SPE) or LC fractionation and characterized by nuclear magnetic resonance spectroscopy (NMR), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high resolution MS. Steroid glucuronides were obtained in milligram amounts with good yields (>85-90%). The synthesised glucuronides were used in method development and as reference material in the analysis.

For the analysis of the steroids in brain hypothalamus, hippocampus, midbrain, and cortex as well as in urine samples, two sample preparation methods were developed; one for brain samples and another for urine samples. The analysis of steroids and their metabolites in brain tissue samples requires an efficient sample preparation as the brain contains high concentrations of lipids, which are difficult to remove from brain extracts. High concentrations of lipids can block an analytical column and cause retention time shifts. Some lipids, especially phospholipids, can interfere analysis by suppressing electrospray ionisation of steroids. Therefore the optimisation of the extraction solvents and purification steps are needed. In the study, homogenization was performed by ultrasound sonicator in ethanol-acetone mixture, and brain extracts were purified with

mixed-mode SPE. Urine samples, on the other hand, contain high quantities of salts and creatine, and although the urine matrix is much simpler than the brain matrix, purification is still needed. For the analysis, two methods were developed, one using capillary liquid chromatography-electrospray-tandem mass spectrometry (CapLC-ESI-MS/MS) and the other using ultra performance liquid chromatography-electrospray-tandem mass spectrometry (UPLC-ESI-MS/MS). In both methods an end-capped C_{18} column and an ammonium acetate buffered acetonitrile-methanol-water gradient was used, and analytes were analysed by selected reaction monitoring (SRM) mode. ESI was used because it is a soft ionisation technique, which enables direct analysis of intact steroid conjugates. The methods were carefully optimized to obtain good selectivity and maximum sensitivity especially for steroid glucuronides. Linear range of 3-4 magnitudes was obtained ($R^2 > 0.996$) with good precision (RSD < 15%). Detection limits of 6-100 pmol/l were obtained for steroid glucuronides, 10-30 pmol/l for steroid sulphates and 0.03-22 nmol/l for free steroids, respectively.

The developed methods were applied to the analysis of neurosteroids and their glucuronide conjugates in mouse brain extracts, to study *in vitro* metabolism of steroids in rat and mouse brain, and to the analysis of free and glucuronide-conjugated steroids during pregnancy. Using the CapLC-MS/MS method steroid glucuronides were observed for the first time in mouse brain. The UPLC-MS/MS method employed in determination of urinary profiles of steroids and steroid glucuronide conjugates steroids during pregnancy. The first morning samples were collected once or twice a week during pregnancy. The concentrations of 11 out of 27 targeted steroids and steroid glucuronides as well as the concentrations of 25 mostly unidentified C_{21}-steroid glucuronides clearly altered during the pregnancy. In general, the concentrations of the steroids and steroid glucuronides gradually increased during pregnancy, decreased rapidly just before or during delivery, and returned to control sample level five days after the delivery.

List of original publications

This doctoral thesis is based on the following publications:

I Sirkku E. Jäntti, Alexandros Kiriazis, Ruut Reinilä, Risto K. Kostiainen, Raimo A. Ketola: Enzyme-assisted synthesis and characterization of Glucuronide conjugates of neuroactive steroids, Steroids 72(3) (2007) 287-296.

II Sirkku E. Kallonen, Anne Tammimäki, Petteri Piepponen, Raattamaa, Helena; Raimo A. Ketola, Risto K. Kostiainen: Discovery of neurosteroid Glucuronides in Mouse Brain, Analytica Chimica Acta 651(1) (2009) 69-74.

III Sirkku E. Jäntti, Anne Tammimäki, Helena Raattamaa, Petteri Piepponen, Risto Kostiainen, Raimo A. Ketola: Determination of Steroids and Their Intact Glucuronide Conjugates in Mouse Brain by Capillary Liquid Chromatography-Tandem Mass Spectrometry. Analytical Chemistry 82(8) (2010) 3168-3175.

IV Sirkku E. Jäntti, Minna Hartonen, Mika Hilvo, Heli Nygren, Tuulia Hyötyläinen, Raimo A. Ketola, Risto Kostiainen: Steroid and Steroid Glucuronide Profiles in Urine during Pregnancy Determined by Liquid Chromatography-Electrospray Ionisation-Tandem Mass Spectrometry. Submitted to Analytica Chimica Acta

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

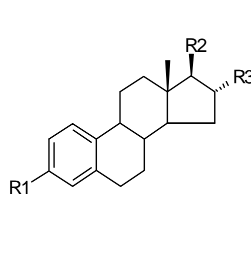
Abbreviations

APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photo ionisation
BBB	Blood brain barrier
BMD	Bismethylenedioxy
CapLC	Capillary liquid chromatography
CD_3OD	Deuterated methanol
CAH	Congenital adrenal hyperplasia
CBG	Corticosteroid-binding globulin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP	Cytochrome P-450 enzyme
ECAPCI	Electron capture atmospheric chemical ionisation
EDTA	Ethylene diamine tetraacetic acid
ESI	Electrospray ionisation
eV	Electron volt
$GABA_A$	γ-amino butyric acid A agonist
GC	Gas chromatography
Glu	Glucuronide conjugate
GP	Girard P reagent
HFBA	Heptafluoro butyric acid anhydride
HMP	2-hydrazino-1-methylpyridine
5-HT	5-Hydroxytryptamine, serotonin
HRMS	High-resolution mass spectrometry
HSD	Hydroxysteroid dehydrogenase enzyme
i.d.	Internal diameter
ISTD	Internal standard
ITMS	Trimethyliodosilane
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass-to-charge ratio
MO	Methoxy amine
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFPH	2-nitro-4-trifuoromethylphenylhydrazine
NH_4Ac	Ammoniumacetate
NMR	Nuclear magnetic resonance
NMDA	*N*-methyl-*d*-aspartate

PAPS	3’phosphoadenosine-5’phosphosulphate
PBS	Phosphate buffered saline
PFB	Pentafluorobentsyl
PCO	Polycystic ovarian disease
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
SHBG	Sex-hormone-binding globulin
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SSI	Sonic spray ionisation
SULT	Sulfotransferase enzyme
TMS	Trimethylsilane
TMSI	Trimethylsilylimidazole
UDPGA	Uridine diphosphate glucuronic acid
UGT	Uridine diphosphate glucuronosyl transferase
UPLC	Ultra performance liquid chromatography

Abbreviations and structures of steroid and steroid conjugates

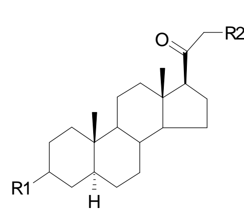
d_5-A-Glu	d_5-5α-androstane-3α-ol-17β-O-glucuronide
AL	Aldosterone;11β,21-dihydroxy-preg-4-ene-3,18,20-trione
AN	Androstendione; androst-4-ene-3,17-dione
AP	Allopregnanolone; 5α-pregnan-3α-ol-20-one
AP-Glu	Allopregnanolone glucuronide
d_4-AP	d_4-allopregnanolone
CORT	Corticosterone; 4-pregnen-11ß,21-diol-3,20-dione
21-CORTG, CORT-21-Glu	Corticosterone 21-glucuronide
CS	Cortisone; 17α-21-dihydroxypreg-4-ene-3,11,20-trione
11-DC	11-deoxycortisol; 17,21-dihydroxypreg-4-ene-3,20-dione
D, DHEA	Dehydroepiandrosterone; 5-androsten-3ß-ol-17-one
DG, DHEA-Glu	Dehydroepiandrosterone 3-glucuronide
DS	Dehydroepiandrosterone 3-sulphate
DHP, 5α-DHP	5α-dihydroprogesterone; 5α-pregnan-3,20-dione
DT	Dihydrotestosterone; 17β-hydroxy-5α-androstan-3-one
E, E2	Estradiol; 1,3,5-estratriene, 3,17 ß-diol
3-EG,E2-3-Glu	Estradiol 3-glucuronide
17-EG, E2-17-Glu	Estradiol 17-glucuronide
E3-3-Glu	Estriol 3-glucuronide
16-ESG, E3-16-Glu	Estriol 16-glucuronide
17-ESG, E3-17-Glu	Estriol 17-glucuronide
HC	Cortisol; 4-pregnen-11ß,17α,21-triol-3,20-dione
21-HCG, HC-21-Glu	Cortisol 21-glucuronide
IP	Isopregnanolone; 5α-pregnan-3ß-ol-20-one
IPG, IP-Glu	Isopregnanolone glucuronide
d_4-NA	d_4-5α-estran-3α-ol-17-one
d_3-5α-N	d_3-17ß-hydroxy-estr-4-ene-3-one
P-Glu	Pregnanolone glucuronide; 5ß-pregnan-3α-ol-20-one Glu
P, PREG	Pregnenolone; 5-pregnen-3ß-ol-20-one
PG, PREG-Glu	Pregnenolone glucuronide
PS	Pregnenolone 3-sulphate
17-OH-PREG	17-hydroxypregnenolone; 3α,17β-dihydroxypreg-5-en-20-one
PRO, PROG	Progesterone; 4-pregnane-3,20-one
T, TES	Testosterone; 4-androsten-17ß-ol-3-one
d_3-TG	d_3-testosterone glucuronide
T, TES	Testosterone; 4-androsten-17ß-ol-3-one
THD, 5α-THDOC	5α-tetrahydrodeoxycorticosterone; 5α-pregnan-3α,21-diol-20-one
3-THDG, 5α-THDOC-3-Glu	Tetrahydrocorticosterone 3-glucuronide
21-THDG, 5α-THDOC-21-Glu	Tetrahydrocorticosterone 21-glucuronide



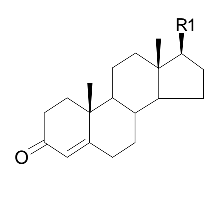
Compound	R1	R2	R3
E2	OH	OH	H
E2-3-Glu	Glu	OH	H
E2-17-Glu	OH	Glu	H
E3-3-Glu	Glu	OH	OH
E3-16-Glu	OH	OH	Glu
E3-17-Glu	OH	Glu	OH

Compound	R1	R2	R3
DHEA	OH	=O	-
DHEA-Glu	Glu	=O	-
DHEAS	SULT	=O	-
PREG	OH	$COCH_3$	H
PREG-Glu	Glu	$COCH_3$	H
PREGS	SULT	$COCH_3$	H
17-OH-PREG	OH	$COCH_3$	OH

Compound	R1	R2	R3	R4
AL	OH	HC=O	CH_2OH	H
CORT	OH	CH_3	CH_2OH	H
CORT-21-Glu	OH	CH_3	CH_2Glu	H
CS	=O	CH_3	CH_2OH	OH
11-DC	H	CH_3	CH_2OH	OH
HC	OH	CH_3	CH_2OH	OH
HC-21-Glu	OH	CH_3	CH_2Glu	OH
PROG	H	CH_3	CH_3	H



Compound	R1	R2
AP	OH (α)	H
AP-Glu	Glu (α)	H
DHP	=O	H
IP	OH (β)	H
IP-Glu	Glu (β)	H
THDOC	OH (α)	CH_3OH
THDOC-3-Glu	Glu (α)	CH_3OH
THDOC-21-Glu	OH (α)	CH_3Glu



Compound	R1
AN	=O
T	OH
TG	Glu

Structure of sulphate is R-O-SO_3H and glucuronide is

Table 1. *The structures of the studied steroids and corresponding sulphate and glucuronide conjugates.*

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1 Review of the literature

1.1 Steroids

1.1.1 Structure, classification and nomenclature

Steroids are endogenous molecules based on a tetracyclic ring structure, with substituents occurring at specific sites within the molecule. They can be classified into five chemical categories based on the estrane (C_{18}), androstane (C_{19}), pregnane (C_{21}), cholane (C_{24}) or cholestane (C_{27}) ring structures. The first three categories are classified into steroids (shown in Figure 1), whereas the two latter categories, cholanes (bile acids) and cholestanes (cholesterols and D-vitamins), are sterols with longer and branched hydrocarbon chain at the C17 position. The nomenclature of the steroid structure is shown in Figure 1. The core ring structure is often substituted and the location of hydroxy-, methyl- or keto-group(s) is most often at positions C2, C3, C4, C6, C7, C11, C12, C16, C17 and C21, and the characteristic double bonds typically occur in the A or B ring. Steroids have several chiral centres, where the orientation of the substituent is either above or below the plane (α- or β-configurations). The pharmacological activities of α/β-isomers usually differ from each other, and in some cases only one of the isomers is active. For example, pregnanolone and tetrahydrodeoxycorticosterone have four known isomers, 3α,5α (allo), 3α,5β (epi), 3β,5α (iso) and 3β,β5 (epiallo), which all have different pharmacological effects.

Steroids can be classified based on their biological activities into estrogens, androgens, progestogens, glucocorticoids and mineralocorticoids, as shown in Figure 2. Classically glucocorticoids and mineralocorticoids are called adrenal steroids, and androstanes, progestogens, and estranes are called sex hormones. Steroids exist in the body tissues and fluids in free form or in conjugation with e.g. glucuronide, sulphate, fatty acid, and amino acid conjugate, or they can be bound to proteins. Bile acids, cholesterols and D-vitamins differ from steroids in their biological functions. The main function of bile acids and their taurine and glycine conjugates are emulsification of nutritional lipids in the gastrointestinal tract that assist in absorption of lipids to the circulation. Cholesterol is a precursor of steroids, and D-vitamins have a role in regulation of calcium and phosphorus levels in the body.

a.

Estrane structure (C18) *Androstane structure (C19)* *Pregnane structure (C21)*

b.

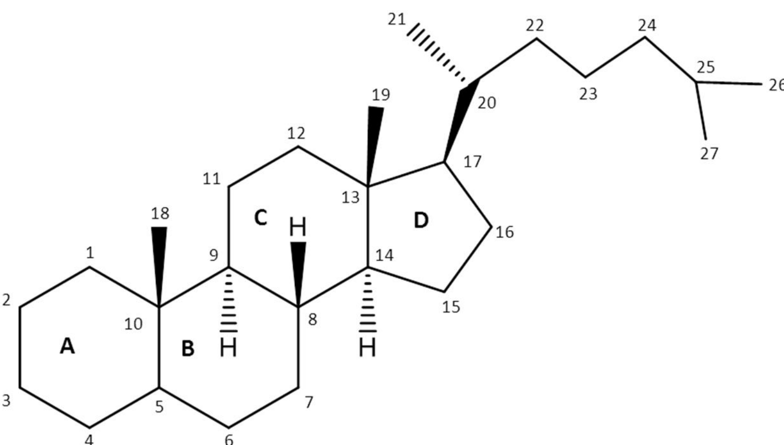
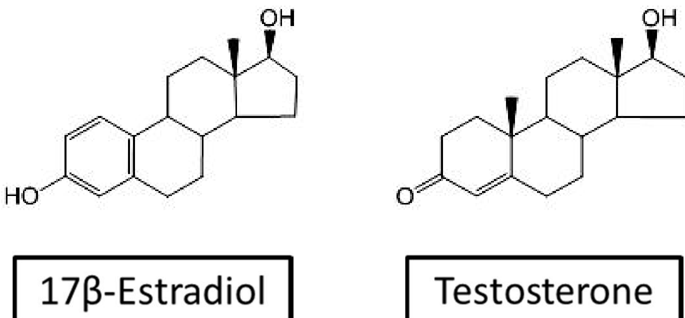
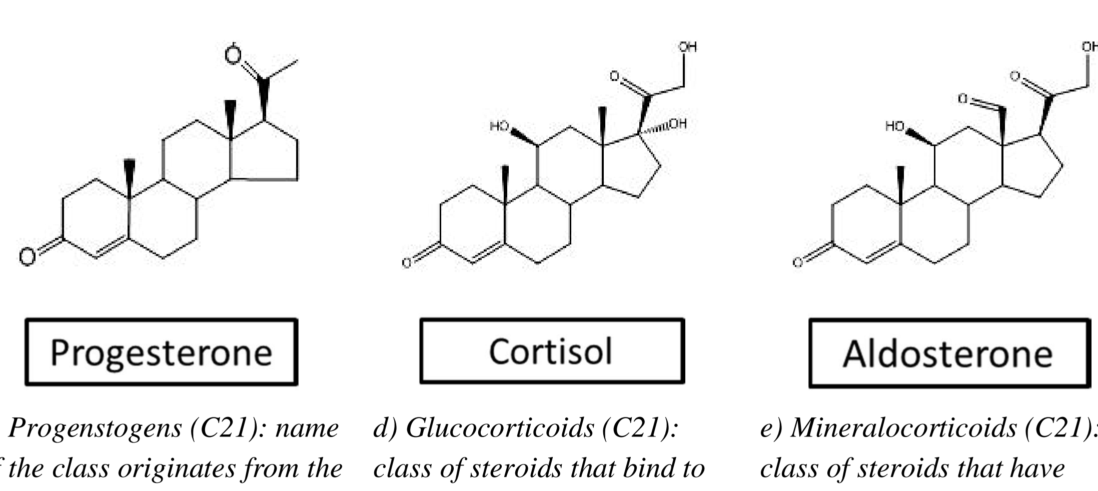


Figure 1. *a) The Classification of steroids into main categories based on ring structure, b) The tetracyclic ring structure of steroids: rings of the steroid skeleton identified by letters and carbon atoms by numbers.*



a) Estrogens (C18): name of the class originates from the importance of the steroids in the estrous cycle.

b) Androgens (C18): class of steroids that bind to the androgen receptors.



c) Progenstogens (C21): name of the class originates from the function to maintaining pregnancy.

d) Glucocorticoids (C21): class of steroids that bind to the glucocorticoid receptor.

e) Mineralocorticoids (C21): class of steroids that have involved in the retention of sodium and a minerals.

Figure 2. *Classification of steroids into main categories based on biological activity by binding to specific receptors (a-e). Structure of the model compound given for each category.*

1.1.2 Physiology

Steroids are synthesised mainly by endocrine glands such as the gonads (androgens and estrogens), the adrenals (corticosteroids, mineralocorticoids and androgens) and during gestation also actively by the placenta (estrogens, progestogens). Secretion organs release steroids into the blood circulation, and they are transferred to target cells, where they are bound to the target receptors. Steroids are relatively lipophilic compounds, and they are found in biological fluids mostly in conjugated form, or they are bound to proteins, for example, to albumin (20-50% of bound fraction), corticosteroid-binding globulin (CBG) or sex-hormone-binding globulin (SHBG) in the plasma. Only the free fraction (1-10% of total steroid concentration in the plasma) is considered to be able to bound to their target locations in the cell. Steroids have an influence on many physiological functions in the body, for example, fetal development, maintaining of salt-water balance, fertility, maintaining pregnancy, and on stress responses as presented in Figure 3. Alterations in normal steroid profiles can provide definitive diagnostic tools for example in enzyme deficiencies [Shackleton et al., 1986], polycystic ovarian disease (PCO) [Stener-Victorin et al., 2010], Cushing's syndrome [Lynette et al., 2002], and congenital adrenal hyperplasia (CAH) [Schwarz et al., 2009].

1.1.3 Steroids in pregnancy

Endogenous estrogens and progestogens have an important role in controlling and maintaining pregnancy. The concentrations of several steroids have been shown to change significantly during pregnancy and postpartum; progesterone and estradiol syntheses are dramatically increased with gestational age and drop sharply after parturition, which seems to be a common trend also to allopregnanolone [Milewich et al., 1975; Stoa et al., 1975; Parker et al., 1979; Albrecht et al., 1990; Pearson Murphy et al. 2001; Parisek et al., 2005]. Also a decrease in progesterone and an increase in estradiole levels in plasma have been observed before the onset of delivery [Nathaniels et al., 1998; Wu et al., 2004]. The role of steroids during pregnancy was recently reviewed by Hill et al. [Hill et al., 2010]. The role of neuroactive steroids during pregnancy and postpartum has not been yet studied in detail, however, the changes of neuroactive steroids can affect mood during pregnancy and postpartum. For example, low levels of allopregnanolone in plasma or cerebrospinal fluid are proposed to be related to prenatal or postpartum depression [Nappi et al., 2001]. The decrease in the levels of allopregnanolone could also trigger the production of oxytocin resulting in a rapid delivery [Brussaard et al., 1998; 1999 and 2000].

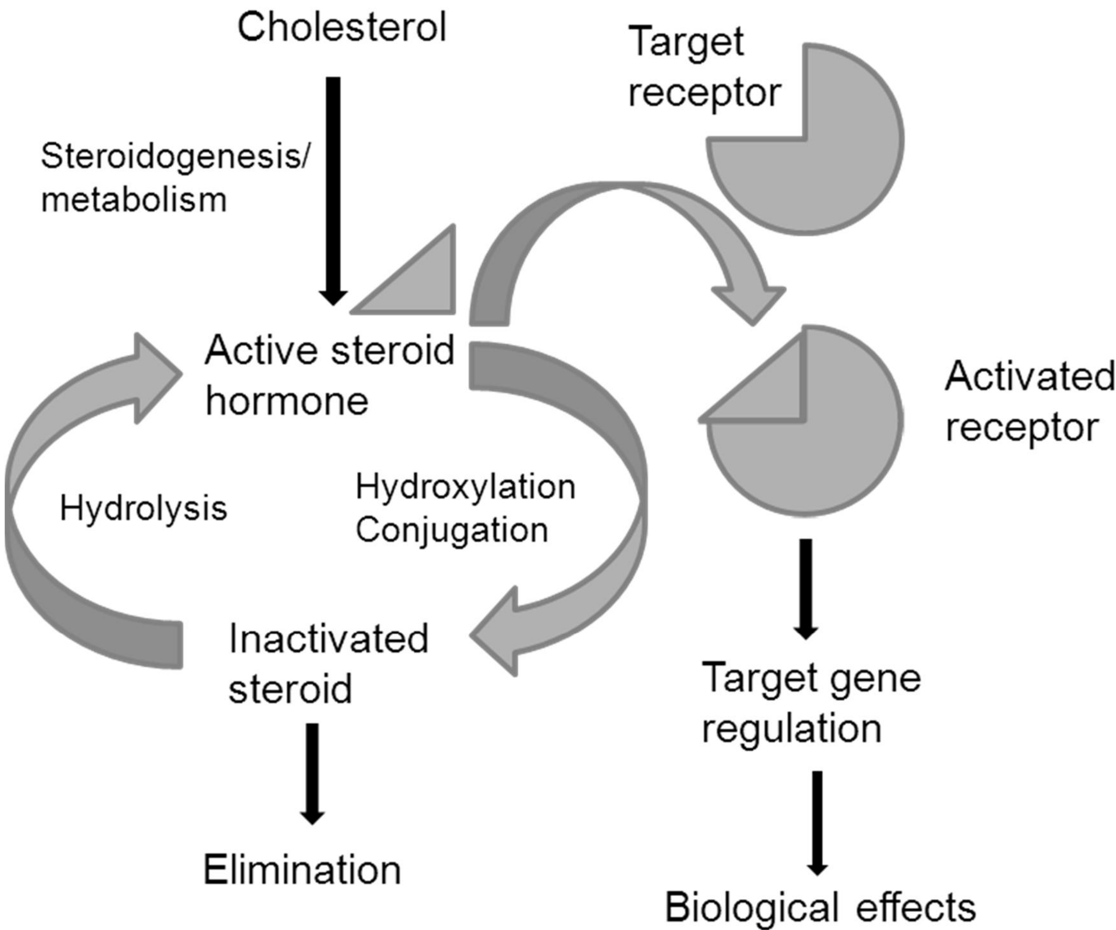


Figure 3. *The active form of a steroid is generated in an organism's metabolism and the steroid activates the ligand-specific steroid hormone receptor by binding. Activation leads to gene regulation and the production of specific proteins generating the biological effects in the body. The system can be maintained with a dynamic balance between formation, inactivation and elimination of the active form. Xenobiotics can disrupt normal steroid physiology by interfering with receptor activation, disrupting steroid production, and altering steroid inactivation. Flow chart modified from You et al., 2004.*

1.1.4 Neurosteroids and Neuroactive Steroids

Since steroids can easily pass the blood/brain barrier (BBB) due to their high lipid solubility, the brain is an important target site of many steroids. Moreover, extensive steroid metabolism occurs in the brain. Neurosteroids can be further divided into two subclasses based on their activity in the brain and on their origin: neurosteroids and neuroactive steroids. Neurosteroids are defined as steroids that accumulate in the brain in the absence of steroidogenic glands and are synthesised in the brain from endogenous precursors by enzymes that are present *in situ* [Baulieu et al., 1990; 1997]. Neuroactive steroids are synthesised by other sources, they disappear from the CNS after removal of steroidogenic glands, and they are not considered as neurosteroids even though they express activity in the brain [Baulieu et al., 1999].

The studies of *de novo* steroid biosynthesis in the brain demonstrate the expression of the key enzymes [Mensah-Nyagan et al., 1999; Compagnone et all, 2000; Tsutsui et al., 2000; Yu et al., 2002; Do Rego et al., 2007, 2009]. Enzymes involved in neurosteroidogenesis are found in several parts of brain as shown in Figure 4. The activity of the steroidogenic enzymes has also been demonstrated with the capability of frog brain tissue to convert deuterium-labelled pregnenolone into 17-hydroxypregnenolone, 17-hydroxy-progesterone, dehydroepiandrosterone and androstenedione metabolites [Do Rego et al., 2007]. It has also been recently shown that pherical pregnenolone levels have only a minor influence on the levels in the cerebrospinal fluid (CSF), which indicates that at least pregnenolone may be synthesised *de novo* from cholesterol in the CNS ($P < 0.05$) [Kancheva et al., 2010]. Though evidence of brain neurogenesis is rather convincing, it still requires further studies. In summary, it seems that substantial part of the steroid metabolites in the CNS may be synthesised in the steroidogenic glands and transported through the BBB either by the diffusion or by transporters. Even though it is evident that steroid metabolism takes place in the brain, still further research is required to clarify the concepts of neurosteroids and neuroactive steroids, as well as for more accurate elucidation of whether the metabolism is mainly secretion route or whether is it also a way to regulate neuroactivity of steroids.

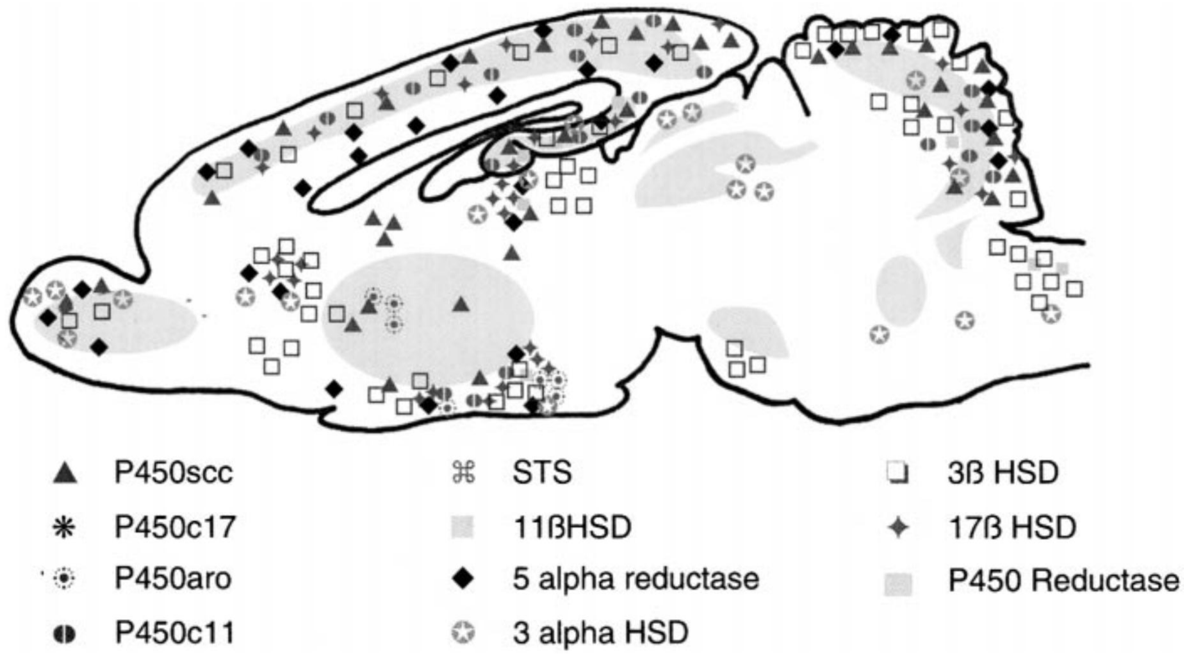


Figure 4. *Schematic representation of an adult brain showing regional expression of enzymes involved in neurosteroidogenesis. Data is collected from several species, including rodent, primates, and amphibians. The symbols for steroidogenic enzymes are shown at the bottom of the figure. All the main enzymes are detected in cortex, hypothalamus and thalamus. Reprint with permission from Compagnone et al., 2000].*

Although the neurosteroidogenesis is not fully understood, neurosteroids and neuroactive steroids have been shown to be involved in the modulation of several receptors, such as γ-amino butyric acid A agonist ($GABA_A$), N-methyl-D-aspartate (NMDA), and sigma receptors [Mellon et al., 2002; Stoffel-Wagner et al., 2003; Belelli et al., 2005; Do Rego et al., 2009]. Several reviews and articles have also described the pharmacological significance of neurosteroids, reporting their involvement in the regulation of a variety of diseases, behavioural and cognitive functions such as anxiety, depression, and aggression, [Jain et al., 1995; Gasior et al., 1999; Mellon et al., 2002; Rupprecht et al., 2003; Pisu et al., 2004] anaesthesia, insomnia, and sleep disorders, [Rupprecht et al., 2003; Gasior et al., 1999; Jain et al., 1995; Majewska et al., 1986] memory, [Rupprecht et al., 2003; Pisu et al., 2004; Jain et al., 1995] stress response, [Mellon et al., 2002] attention deficit hyperactivity disorder (ADHD), [Rupprecht et al., 2003] cancer, [Jain et al., 1995] schizophrenia and bipolar disorder, [Marx et al., 2005] as well as Alzheimer's and Parkinson's diseases. [Schumacher et al., 2003; Weill-Engerer et al., 2002].

1.2 Metabolism of steroids

1.2.1 Biosynthesis of Steroids (phase I metabolism)

Steroids are synthesised from cholesterol, which is supplied from different sources. Cholesterol can be synthesised from acetyl coenzyme A by enzymes in the cellular microsomes and cytosol, or it can be supplied by hydrolysis of esterified cholesterol stored within cells or released by plasma low density lipoproteins. Enzymes responsible for transformation of cholesterol to steroids are mainly classified into two major categories of proteins: the cytochrome P450 (CYPs) and hydroxysteroid dehydrogenases (HSDs). CYP enzymes, using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, typically catalysed oxidation reactions with steroid molecule resulting in hydroxylation products and cleavage of the cholesterol side chain. HSDs are enzymes, which transform hydroxyl groups to oxo-groups using nicotinamide adenine dinucleotide (NAD^+) or its phosphate ($NADP^+$). CYPs and HSDs, together with 5α/β-reductases, are the main enzymes responsible for hormone steroid metabolism, which take place in the cell mitochondria and microsomes. Reductase enzymes are responsible for reducing the double bond in a ring structure of 3-oxo-4-ene steroids. The most common inactivating reactions of steroids involve the irreversible reduction of keto- function (e.g. present at C3, 11 and 20) and alkene groups present in the core structure [Andrew, 2001].

At the first steps of steroid biosynthesis, pregnenolone is formed from cholesterol, and secondly it is metabolised to progesterone and 17-hydroxypregnenolone as shown in Figure 5. Progesterone is further transformed to the glucocorticoid (yellow area in Figure 5), mineralocorticoid (orange area) and pregnane metabolites (white area), and both progesterone and 17-hydroxypregnanolone are further transformed via androgen-estrogen pathways (green and blue area) as presented in Figure 5. Estrogens are formed from androgens by aromatase enzyme, a member of the CYP enzyme family.

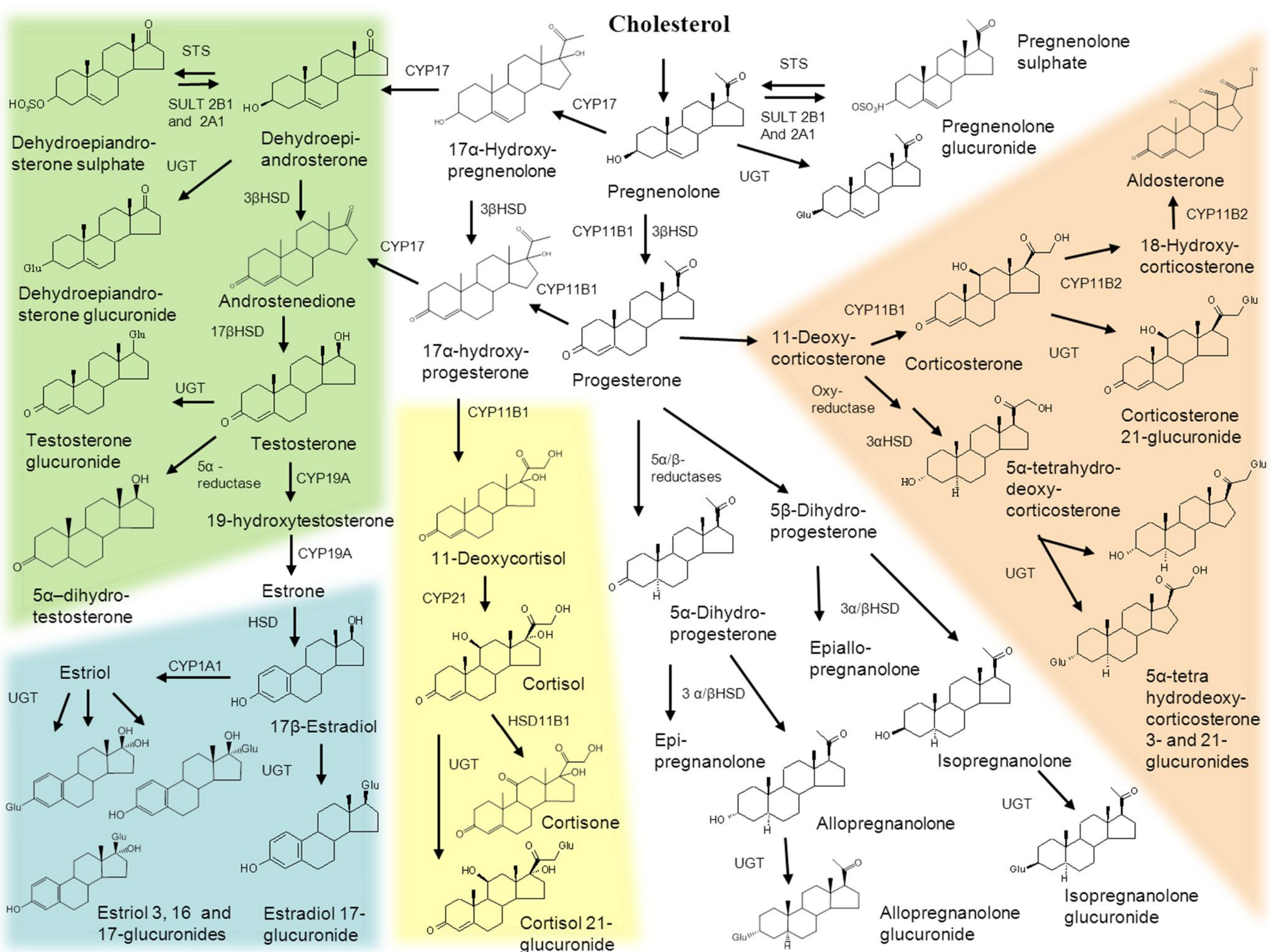


Figure 5. *Biosynthesis route of steroids [Andrew,2001; Fuda et al., 2002; Mensah-Nygan et al., 1999; Stoffel-Wagner, 2001; Mellon et al., 2002; Payne et al, 2004; Kickman, 2010; www.kegg.jp]. The steroid pathway is divided between androgen-estrogen (green and blue area), glucocorticoid (yellow area), mineralocorticoid (orange area) and progestogens metabolites (white area).*

1.2.2 Conjugation reactions (phase II metabolism)

Phase I metabolism of the steroids produces hydroxyl metabolites, which are further substrates for conjugation reactions (phase II metabolism). Conjugates are more water-soluble and have lower affinity to plasma proteins, and conjugation promotes steroid excretion to urine and bile. Conjugates are generally inactive, although deconjugation is possible and thus conjugates remaining in the circulation can be regarded as a pool of steroid. [Andrew, 2001] Conjugates can also be pharmacologically active, e.g. sulphate-conjugated pregnanolone and dehydroepiandrosterone can affect neuronal activity via the modulation of sigma 1 receptors, GABAergic, and glutamatergic neurotransmission [Akwa et al., 2000; Schumacher et al., 2008].

Glucuronidation is one of the most common conjugation reactions, and it is also a major conjugation route for steroids. Uridine diphosphate glucuronosyl transferases (UGTs) are membrane-bound enzymes of the endoplasmic reticulum, and they catalyse the attachment of the glucuronic acid to the hydroxyl-, amine-, thiol- and carboxylic acid groups. Reaction uses uridine diphosphate glucuronic acid (UDPGA) as the co-substrate as presented in Figure 6. According to current knowledge UGT family contains 117 individual UGTs [Dalvie et al., 2012] of which at least 25 UGTs are found in humans [Tukey et al., 2000; Mackenzie et all. 2005]. UGT isoenzymes have been categorized into four families: UGT1, UGT2, UGT3 and UGT8 on the basis of the protein sequence similarity [Tukey et al., 2000, 2001; Mackenzie et al., 2005; Radominska-Pandya et al., 2005; Meech et al., 2010], and the enzyme families are further divided into subfamilies according to their sequence homology [Burchell et al., 1991; Mackenzie et al., 1997]. Most UGTs are expressed in the liver, which is considered to be the major site of glucuronidation. However, many of the UGTs are also found in other tissues as summarised in Table 2. UGTs 1A6 [King et al., 1999; Suleman et al., 1998], 2B7 [King et al., 1999] and 2B19 [Bélanger et al., 1999] have been found in human, rat or monkey brain. Steroids are reported as substrates of several UGT1 and UGT2 isoenzymes (Table 2), and the most important enzymes involved in human steroid glucuronidation are members of subfamilies UGT1A and UGT2B [Hum et al., 1999]. The extent of glucuronidation varies with steroid type: greater than 90% of tetrahydrodeoxycorticoids being excreted as glucuronides, whereas cortisol glucuronide comprises less than 50% of the total cortisol in urine [Best et al., 1997].

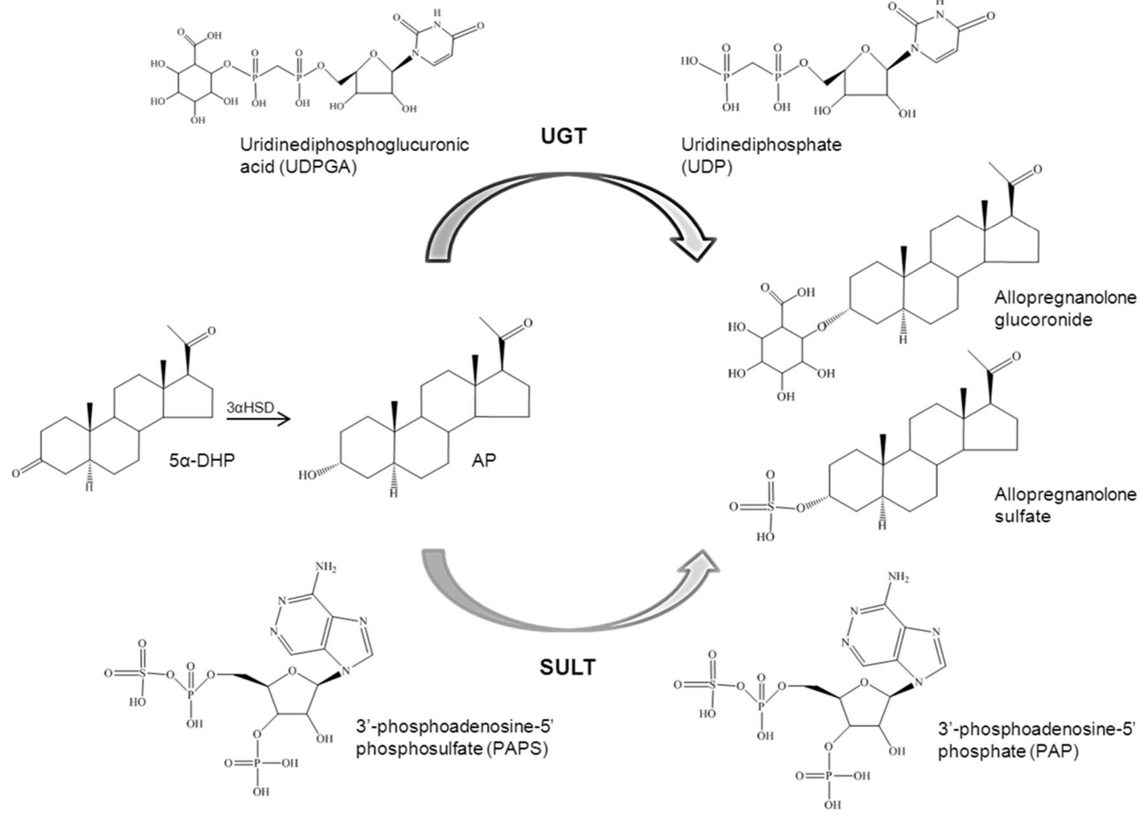


Figure 6. *The conjugation reactions of steroids: Phase I metabolism serves sites for conjugation; hydroxyl groups of allopregnanolone are further glucuronidated by UGT enzymes and/or sulphated by SULT enzymes.*

Another important conjugation reaction of steroids is sulphate conjugation. Sulphotransferases (SULTs) are mostly cytosolic enzymes that attach a sulphate group to an O-, N- or S- acceptor group using 3'phosphoadenosine-5'phosphosulphate (PAPS) as a co-substrate as presented in Figure 6. SULTs are divided according to their amino acid sequence and enzyme function to four families: SULT1, SULT2, SULT4 and SULT6. At least 13 distinct members of SULT families have been identified [Lindsay et al, 2008]. Isoenzymes SULT 2A1, SULT 2A2, and SULT 2B1b isoenzymes have been found in the rat, human, or monkey brain [Falany et al, 2000; Sakakibara et al., 2002; Shimizu et.al, 2002, 2004; Shimada et al, 2001], and SULT 2B1, SULT 2A1 and SULT1E1 are known to sulphate steroids [Comer et al. 1993; Fuda et al., 2002; Gamage et al., 2006].

Table 2. *List of isoenzymes in the human UGT1 and UGT2 family, their tissue distribution and reported steroid substrates: A; androgens, E; estrogens, P; pregnanes.*

UGT isoenzyme	1A1	1A3	1A4	1A5	1A6	1A7	1A8	1A9	1A10	2A1	2A2	2B4	2B7	2B10	2B11	2B15	2B17	2B28	2B30
Substrates [A]	E	A E	A E P			E	A E	E	A E	A E	E	E	A E P		A	A E	A E P	A	A E P
Adrenal gland																	X		
Biliary tract/tissue	X	X	X		X				X										
Brain					X					X			X						
Colon	X	X	X		X	X	X	X	X				X						
Intestine	X		X	X			X		X			X	X	X	X	X			
Kidney	X	X			X		X	X	X			X	X	X	X	X	X		X
Liver	X	X	X		X			X				X	X	X	X	X	X	X	
Lung						X				X		X	X	X	X	X	X		
Mammary gland					X			X				X	X	X	X	X	X	X	
Pancreas	X	X	X	X		X	X	X	X										
Placenta																			
Prostate		X						X				X		X	X	X	X		
Skin					X			X									X		
Stomach	X	X		X	X	X		X	X										
Testis		X										X		X		X	X		

A; androgens, E; estrogens, P; pregnanes. References: Basu, 2004; Beaulieu, 1996; Bélanger, 1998; Bowalgaha, 2007; Cheng, 1999; Coffman, 1998; Dalvie, 2012; Finel, 2005; Gall, 1999; Green, 1994; 1998; Green, 1996; Girard, 2002; 2003; Hum, 1999; Itäaho, 2008; Jin, 1993; 1997; King, 1996; 2000; Lepine, 2004; Lévesque, 2001; Mojarrabi,1996; Strasburg, 1998; StarlardDavenport, 2008; Tukey, 2000; Turgeon, 2001; Zhang, 2007.

1.3 Analysis of steroids and their conjugated metabolites

Analysis of steroids and their conjugates in biological tissues is demanding due to the relatively low concentration levels of steroids in a complex biological matrix. Thus, the total analytical work flow must be carefully designed including the selection of correct biomatrix, sampling, sample preparation and analysis.

1.3.1 Extraction, isolation and purification

Sample preparation is usually needed in the bioanalytical determinations. The steps of the procedure are typically homogenization of samples, extraction, and purification of the extract. The extraction of steroids is typically done by using organic solvent. Purification is almost always required, because biological samples contain large number of compounds causing matrix effects in the analysis. The purification is most commonly done using liquid-liquid extraction (LLE) or solid phase extraction (SPE). The advantage of LLE is simplicity, but it is a quite rough and non-specific purification method, which is not capable of separating similar types of compounds. SPE is more specific method and can be easily automated, but it requires more method development and is more time consuming technique than LLE. Tissue samples, such as brain biopsies, are complicated samples, and they contain a large amount of lipids, which makes the purification steps mandatory. High amounts of lipids in the brain tissue extracts can block an LC column and cause shifts in retention times. Some lipids may also suppress the ionisation of steroids in LC-MS analysis. Many of the current methods in steroid analysis of brain tissue samples include several sequential purification steps by SPE (combination of ion exchange and C18 cartridge) or LC fractionation step (Table 3). Analysis of steroids in biological fluids, such as plasma, serum, urine and CSF demands at least the removal of proteins or salts prior to the analysis, for example, using protein prepicitation, SPE, LLE, or on-line column switching techniques. Examples of preparation procedures for the analysis of steroids in biological matrices are given in Table 3.

Table 3. *Examples of analyses of endogenous steroids from tissues and biological fluids by chromatographic methods coupled to mass spectrometer.*

Steroid analytes	Matrix	Technique	Sample preparation	Limit of Detection/ Quantitation	Analysis of conjugates	References
46 steroids: androgens, corticoids, pregnanes	Brain, rat	GC-MS	Homogenization (PBS), LLE, SPE (Oasis HLB, MCX), derivatisation (MO-TMSI, HFBA)	LOD 0.004-1.0 ng/g for steroids LOD 0.04-1.5 ng/g for steroid sulphates	Hydrolysis (chemical)	Ebner et al., 2006
4 steroids: androgens, pregnanes	Brain, rat	GC-MS	Homogenization (MeOH:H_2O), SPE fractionation (C18) Steroids: LC fraction collection prior analysis, derivatisation (HFB) Sulphates: solvolysis, SPE (C18)	LOD 1-5 pg/ injection	Hydrolysis (chemical)	Liere et al, 2000
5 steroids: androgens, pregnanes	Brain, rat	GC-MS	Homogenization (75 % MeOH), SPE (C18), derivatisation (PFB)	LOD 0.25 ng (in 100mg tissue)	No	Vallée et al., 2000
10 steroids: androgens, estrogens, pregnanes	Brain, rat	LC-APCI-MS/MS	Homogenization (MeOH:Acetic acid), SPE (C18)	LOQ 0.02-0.25 pg/sample	No	Caruso, et al., 2008
3 pregnanes, 2 androgens	Brain, rat	LC-ESI-MS/MS	Homogenization (MeOH:Acetic acid), SPE (Strata X), derivatisation (HMP)	Pregnanes: LOQ 0.25 ng/g, Androgens: LOQ 0.05-0.1 ng/g	No	Higashi et al., 2007, 2008
2 pregnanes	Brain, rat	LC-ECAPCI-MS/MS	Homogenization (1%AcOH in MeOH), SPE (Oasis HLB, Bond Elut Si), derivatisation (NFPH)	LOD 1-6 pg/ sample	No	Higashi et al., 2005

7 steroids: androgens, pregnanes	Brain, rat	CapLC-ESI-MS/MS	Homogenization (EtOH), SPE (C18, cathion exchange, anion exchange). Steroids: derivatisation (oximes), SPE (C18, cathion exchange); Sulphates: SPE (C18)	LOD 0.1-3 pg/ injection	Direct analysis (2 Sult)	Liu et al., 2003
18 steroids: androgens, pregnanes, cortisol	CSF, human	GC-MS	LLE, derivatisation (methoxylamine-hydrochloride, TMS-MOX)	LOD 0.04-11 pmol/L (0.6-62 fg)	Hydrolysis (chemical and β-glucuronidase)	Kancheva et al., 2010
6 steroids: androgens, pregnanes	CSF, human, monkey	GC-MS	SPE, derivatisation (carboxyloxime, PFB, TMS)	LOD ~2-15 pg/ mL	No	Kim et al. 2000
65 steroids: all classes	Plasma, human	GC-MS	SPE, LLE, derivatisation (TMS)	LOQ 0.2-2 ng/ mL	No	Ha et al., 2009
6 steroids: androgens, pregnanes	Plasma, human, rat	GC-MS	SPE, derivatisation (carboxyloxime, PFB, TMS)	LOD ~2-15 pg/ mL	No	Kim et al., 2000
5 steroids: androgens, pregnanes	Plasma, rat	GC-MS	SPE (C18), derivatisation (PFB)	LOQ 0.1 ng/ 300 µL of albumine sol.	No	Vallée et al., 2000
3 corticoids	Plasma, human	GC-MS	SPE (Sep-Pac C_{18}), derivatisation (BMD-PFP)	NA	No	Furuta et al., 1998
18 steroids: androgens, pregnanes, corticoids	Serum, human	GC-MS	LLE, derivatisation (methoxylamine-hydrochloride, TMS-MOX)	LOD 0.04-11 pmol/L (0.6-62 fg)	Hydrolysis (chemical and β-glucuronidase)	Kancheva et al., 2010
6 steroids: androgens, estrogens	Serum, mouse, horse, baboon, sheep	LC-APPI-MS/MS	Homogenization (EDTA in PBS), LLE, on-line SPE	LOQ 0.5-40 pg/ injection (corresponding 0.01-0.6 ng/mL)	No	McNamara et al., 2010
2 estrogens	Serum, human	LC-ESI-MS/MS	LLE, SPE (Strata X)	LOD 0.005 ng/mL	Direct (5 Glu, 1 sult)	Caron et al., 2009

Steroid analytes	Matrix	Technique	Sample preparation	Limit of Detection/ Quantitation	Analysis of conjugates	References
4 estrogens	Serum, human	LC-ESI-MS/MS	Protein precipitation	LOD 0.001-0.002 ng/mL	No	Guo et al, 2008
8 estrogens	Serum, human	LC-ESI-MS/MS	LLE, derivatisation (dansyl chloride)	LOQ 8 pg/mL 0.4 pg/injection	Hydrolysis (β-glucuronidase and sulphatase)	Xu et al., 2007
12 steroids: all classes	Serum, human	LC-APPI-MS/MS	Protein precipitation	LOD 0.001-0.01 ng/mL	No	Guo et al., 2006
9 steroids: all classes	Serum, human	LC-APCI-MS/MS	Protein precipitation, on-line SPE	LOQ 0.05-48 ng/mL	Direct: DHEAS	Ceglarek et al. 2009
30 steroids: androgens, corticoids, pregnanes	Urine, human 24-h	GC-MS	SPE, derivatisation (MO-TMS)	NA	Hydrolysis (helix pomatia)	Chan 2008
3 corticoids	Urine, human	GC-MS	SPE (Sep-Pak C_{18}), derivatisation (BMD-PFP)	NA	No	Furuta et al., 1998
11 androgens	Urine, human	GC-MS	SPE, derivatisation (ITMS/MSTFA)	-	Hydrolysis (chemical and β-glucuronidase)	Dehennin et al., 1996
23 steroids: estrogens, androgens, pregnanes, corticoids	Urine, primate	LC-ESI-MS/MS	LLE	LOQ 0.3-3 ng/mL	Hydrolysis (chemical and β -glucuronidase)	Hauser et al., 2008
5 androgens	Urine, human	LC-MS/MS	SPE (WAX)	LLOQ 0.4-100 ng/mL	Direct analysis (sult)	Strahm et al., 2008

5 steroids: estrogens, pregnanes	Urine, human	LC-MS/MS	Automated SPE (C18)	LOQ 6-61 pg on-column (load vol. 5-50 mL)	Hydrolysis (β-glucuronidase)	Ălvarez Sanchez et al., 2008
5 androgens	Urine, Human, bovine	LC-MS/MS	SPE (Strata X)	LOD 80-100 ng/mL	Direct analysis (sult, glu)	Biuarelli et al., 2004
2 corticoids	Urine, human	LC-MS/MS	LLE	LOQ 25-30 ng/mL	No	Taylor et al., 2002
7 androgen conjugates	Urine, human	LC-SSI-MS	SPE	LOQ 10-80 ng/mL	Direct analysis (sult, glu)	Jia et al.. 2001
6 steroids: androgens, estrogens	Tissue (testis, prostate, ovary, uterus), mouse	LC-APPI-MS/MS	Homogenization (EDTA in PBS), LLE, on-line SPE	LOQ 0.5-40 pg/ injection	No	McNamara et al., 2010
15 estrogens, testosterone	Tissue (lymph node; breast carcinoma), human	CapLC-ESI-MS/MS	Homogenization (NH_4HCO_3), LLE, derivatisation (dansyl chloride)	-	Hydrolysis (β-glucuronidase and sulphatase)	Blonder et al., 2008
5 steroids: androgens, estrone	Tissue (adipose), human	GC-MS	Homogenization (liq.N_2), LLE, SPE	-	No	Bélanger et al., 2006
2 corticoids	Tissue (liver) mouse	LC-ESI-MS/MS	Homogenization ($MeOH:H_2O$), LLE	LOQ 60 nmol/kg	No	Rönquist-Nii et al., 2005
4 corticoids	Tissue (adipose), human, mouse	LC-ESI-MS/MS	Homogenization (ethyl acetate), LLE	LOQ 0.075-6 nmol/kg	No	Rönquist-Nii et al., 2005

Abbreviations see chapter Abbreviations.

The choice of an analytical technique sets also the requirements for the sample preparation methodology. For example, derivatisation of steroids is usually necessary before GC-MS analysis and steroid conjugates are not able to measure as intact form, and enzymatic or chemical hydrolysis is needed. These additional sample preparation steps are prone to errors. For example, hydrolysis can alter or decrease the information obtained from the analysis: the origin of a conjugate can be ambiguous, the information of the conjugation site(s) is lost, and incomplete hydrolysis is possible.

It has been suggested that steroid sulphates, detected with indirect methods from brain samples, are actually other conjugates such as steroid glucuronides [Liu et al., 2003] or fatty acid ester conjugates [Liere et al., 2004]. Several research groups have also noticed that neurosteroid sulphate levels obtained by RIA method without hydrolysis [Coperchot et al. 1981, 1983], cannot be reproduced by direct methods or methods including more specific sample purification, which exclude the effect of steroids originating from other types of conjugates [Liere et al., 2004; Mitamura et al., 1999; Liu et al. 2003; Schumacher et al., 2008; Liere et al., 2009]. Thus, conclusions based on indirect analysis may be inaccurate, or even erroneous. Therefore, direct analytical methods using LC-MS are preferred over the indirect methods such as GC-MS or RIA.

1.3.2 Analytical methods

RIA and GC-MS. The methods previously used for steroid assays were mainly based on RIA and GC-MS. The advantage of RIA is a rapid throughput of samples. The antibodies are available commercially for the most important steroids. RIA is sensitive (7-15 pg), but the lack of selectivity and cross reactions are major disadvantages [Corpéchot et al., 1993]. The disadvantage arises from the close structural similarity between many steroids found in biological fluids, and insufficient specificity of antibodies to differentiate these structures from each other. For that reason RIA often overestimates the steroid concentrations. For example, in the study where methods based on commercial immunoassay kits were compared to isotope dilution GC-MS, seven of ten immunoassays overestimated the level of testosterone in female serum samples [Taieb et al., 2003]. The average concentration obtained with immunoassay was 46% higher than with GC-MS. If the cross reactivity of immunoassay is not studied, origin of the metabolites may remain partially unclear, and uncertainties can lead to controversial results. The more complicated the sample, the more difficult, expensive and laborious is the measurement of all relevant cross reactivities. For example, using a RIA-method the concentrations levels of 1-10 ng/g of steroid sulphate conjugates were measured in the brain, [Coperchot et al. 1981, 1983], whereas these sulphates are found at essentially lower level or not detected at all using more specific methods [Liere et al., 2004, Mitamura et al., 1999, Liu et al. 2003]. Other disadvantages are that RIA may require relative large sample volumes, when steroids are present at low concentrations and use of radioactive labels restricts use of RIA.

GC-MS is a robust and widely used method for steroid bioanalysis with high sensitivity and selectivity. The structural and α/β-stereoisomers of steroids can be separated easily, and detection limits (LODs) of 0.3-1.5 ng/g (1-5 pg) have been obtained in the analysis of steroids in brain tissue samples [Liere et al., 2000, Valée et al., 2000], and pg/mL to ng/mL range in urine, blood and CSF (Table 3). The disadvantages of the methodology are that steroid conjugates have to be hydrolysed prior to analysis and derivatisation of the polar functional groups in steroids is usually mandatory to achieve evaporation and adequate chromatographic properties of the analytes for GC-MS analysis. Hydroxyl groups of a steroid molecule can be transformed, for example, to trimethyl silyl (TMS) and tert-butyldimethyl silyl (TBDMS) derivates, and a carboxyl group to methoxyether (MOX) derivates. The need for hydrolysis and derivatisation complicates the analysis and increases analysis times. Derivatisation of steroids with several oxo- and hydroxyl groups in the structure, for example cortisol and corticosterone, can lead to several chromatographic peaks, and that way complicates quantification or interpretation of the results.

LC-MS. LC-MS provides good separation efficiency, high sensitivity and selectivity. Separation efficiency is good especially when ultra performance LC (UPLC), capillary-LC or nano-LC is used. The structural and α/β-stereoisomers of steroids can be separated relatively easily with LC, and both conjugated and free steroids can be analysed simultaneously. LC combined with electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), or atmospheric photo ionisation (APPI) mass spectrometry have been shown to be a very useful methods for the analysis of steroids and their metabolites (Table 3).

ESI is a soft ionisation method, where analytes are ionized by ion evaporation process or by ion-molecule reactions in a gas phase. [Kostiainen et al., 2009] (see Table 4). ESI provides efficient ionisation for steroids having relatively high proton affinity i.e. for steroids having the 3-oxo-4-ene group (eg. many of androgens, gluco- and mineralocorticoids). Furthermore, steroids having an ionic group can be efficiently ionized by ESI (eg. Some of estrogens and glucuronide and sulphate conjugates). For less polar steroids with low proton affinity, such as allopregnanolone and isopregnanolone, the ionisation efficiency of ESI may be poor. However, the ionisation via ammonium adduct formation can be used to enhance the ionisation of steroids having low proton affinity. Achieving high sensitivity with ammonium adducts demands careful optimisation of spray parameters, the temperature of ion source and the flow rate of nebulizing gas. Derivatisation of steroids is also commonly used to improve ionisation efficiency with ESI, but this approach complicates sample preparation resulting in increased analysis times. In general terms, ESI as a soft ionisation method providing direct analysis of steroid conjugates and therefore it is the only ionisation technique that is suitable for simultaneous analyses of steroids and their glucuronide and sulphate conjugates. However, steroid glucuronides or sulphates are quite seldom analysed by LC-MS without a hydrolysis step, most obviously due to the lack of commercially available reference material (Tables 3 and 5).

LC-ESI-MS/MS has been successfully applied to the analysis of many steroids in biological matrices. The methods provide high sensitivity and LODs from pg/mL to ng/mL levels have been achieved (Table 3). For example, Guo et al. measured 4 estrogens

from human serum with LOD of 1-2 pg/mL [Guo et al. 2008], and Hauser et al. 23 steroids and their conjugates after hydrolysis from urine with LODs of 0.3-3 ng/mL [Hauser et al., 2008]. However, the most sensitive current LC-MS methods for the analysis of neurosteroids are based on derivatisation, which can improve the sensitivity 20 - 150-fold compared to the analysis of steroids without derivatisation. The most common derivatisation agents used in the analysis of brain steroids are Girard P reagent (GP), [Griffiths et al., 2003, 2004] 2-nitro-4-trifuoromethylphenylhydrazine (NFPH), [Higashi et al., 2005] and 2-hydrazino-1-methylpyridine (HMP) [Higashi et al., 2005, 2006]. In the analysis of derivatised steroids in brain tissue by LC-ESI-MS/MS, LODs below 1 ng/g have been achieved (see Table 3). In general, for steroids having a 3-oxo-4-ene structure, quite similar LOQs have been reported with derivatisation (1-100 pg/mL) and without derivatisation (1-60 pg/mL) [Matsui et al., 2009, Koal et al., Yamashita et al., 2007, Star-Weinstock et al., 2012, Higashi et al., 2007]. For other steroids derivatisation clearly improves the sensitivity of the method. For example for DHEA, LOQ of 120 pg/mL is reported for the free steroid, and of 25 pg/mL using derivatisation [Higashi et al., 2007, Goal et al, 2012], and for β-estradiol 2-30 pg/mL and 0.5-10 pg/mL, respectively. [Guo et al., 2008, Koal et al., 2012, Weidong et al., 2012, Yamashita et al, 2007] Although derivatisation improves the sensitivity of method significantly, it also precludes the direct analysis of glucuronide conjugates and complicates the method.

APCI, and especially APPI, provide efficient ionisation for non-polar in addition to polar compounds and have been used as alternative ionisation methods to ESI. In APCI ionisation is initiated by a corona discharge needle, and in APPI by 10 eV photons (see Table 4). In both methods ionisation of analytes takes place either by proton transfer or by charge exchange reaction in the gas phase. LC-APPI-MS/MS has been used to measure steroids from human and mammal serum; McNamara et al. measured six steroids with LODs of 10-60 pg/mL [McNamara et al., 2010] and Guo et al. described the analysis of twelve steroids with LODs of 1-10 pg/mL [Guo et al., 2006]. LC-APCI-MS/MS has used for analysis of nine steroids with LOD of 0.05-48 ng/mL from human serum [Calarek et al., 2009].

In general, APPI provides better sensitivity for lower proton affinity and less polar steroids (e.g. pregnanolone), than APCI and ESI, whereas for steroids having higher proton affinity, such as those with 3-oxo-4-ene structure (e.g., testosterone), the sensitivity is the same for APPI and ESI (LOQs of 1-10 pg/mL), and the reported LOQs are a little higher with APCI (LOQs of 60-500 pg/mL [Matsui et al., 2009, Koal et al., 2012, Guo et al., 2006, Zhang et al., 2011, Wang et al., 2012, Reddy et al., 2007, Rauh et al., 2006, Draisci et al., 2000]. β-Estradiol, an example of steroids having an aromatic ring in the structure, behaves in a similar manner as testosterone (LOQ of 1.5-30 pg/mL in ESI and APPI, and LOQ of 60 pg/mL in APCI) [Guo et. al, 2008, Koal et al., 2012, Guo et al., 2006, Zhang et al., 2011, Ceglarek et al., 2009], while steroids having lower proton affinity, for example DHEA, LOQs obtained with APPI are lower (LOQ 1.5 pg/mL) [Guo et al., 2006] than with ESI (LOQ 120 pg/mL) [Koal et al., 2012]. However, results obtained from different studies are not directly inter-comparable due to differences in matrices, sample preparation, and in the LC-MS instruments used. The disadvantage of APCI and APPI is the heat and thermo-labile properties of steroid conjugates, which are easily dissociated in the ionisation process. Consequently, the conjugated species produce

a protonated aclycone and resulting in similar mass spectra as the aclycone itself and therefore, decreased specificity and sensitivity of the analysis.

Analysis of steroids in brain samples. The GC-MS and LC-MS methods in analysis of steroids in brain samples have been summarised in Table 3. In analysis of forty six steroids and steroid sulphates in rat brain samples using GC-MS, LODs of 2.5-1000 pg/g were obtained for the steroids after derivatisation, and 40-1500 pg/g for the steroid sulphates after hydrolysis and derivatisation [Ebner et al, 2006; Vallée et al., 2000]. All together thirteen pregnanes, two androgens and DHEAS could be detected. The most abundant compounds were CORT (4.63±1.0 ng/g tissue), TES (2.24±0.95 ng/g) and PREG (1.67±0.45 ng/g), as others were found at 1 ng/g or lower levels. Vallée's group measured the frontal cortex of control rats and stressed rats after a 10-min swim test by GC-MS. TES, DHEA, PREG, AP and epiAP were found at levels <0.5 ng/g tissue in normal rats, and <7 ng/g tissue in stressed rats. As the concentrations of steroids in brain tissue samples are very low, derivatisation is necessary in order to improve sensitivity of LC-MS. After derivatisation and using LC-ESI-MS/MS, LODs of 50-250 pg/g were achieved for selected steroids [Hitasgi et al. 2007; 2008; Liu et. al 2003]. Higashi et al. measured the concentrations of steroids in brain tissue samples of stressed and control rats by LC-ESI-MS/MS after HMP derivatisation of the steroids. They found that the concentrations of AP, epiAP, and 5α-DHP were higher in stressed rats (0.2-5 ng/g) than in control rats (below LOQ) [Higashi et al., 2007]. Higashi's group also showed, using LC-ECAPCI-MS, that pregnenolone (PREG) and progesterone (PROG) levels were significantly higher in stressed rat brains (5-70 ng/g and 0-22 ng/g, respectively) than in control rat brains (PREG: 5-10 ng/g, PROG: < LOD) [Higashi et al., 2005]. Liu et al. detected 0.04-38 ng/g of neurosteroids keto-structure as their oxime derivatives in rat brain extracts using nano-LC-ESI-MS/MS [Liu et al., 2003]. They also applied the method for a direct analysis of PREG and DHEA sulphates, but no sulphates were found (LODs were 0.3 ng/g). Steroid glucuronides have not been found in brain tissue samples earlier by direct or indirect methods, most obviously due to the lack of reference compounds and sufficiently sensitive and selective methods. Very recently our findings of steroid glucuronides in brain tissue have been confirmed using an indirect LC-ESI-MS/MS method with enzymatic hydrolysis [Maeda et al., 2013].

Table 4. *Comparison of ionisation techniques in LC-MS analysis of steroids.[Kostiainen et al., 2011; Marchi et al,. 2009]*

	ESI	**APCI**	**APPI**
Schematic drawing of the ion source	Nebuliser gas; Spray capillary at high voltage; Orifice of MS; LC eluent; Spray	Auxiliary gas; Orifice of MS; Heater; LC eluent and nebuliser gas; Corona dischange needle	Auxiliary gas; N2; UV lamp emission of photons (10 eV); Orifice of MS; Heater; LC eluent and dopant
Main principle	The eluent is sprayed onto the fine charged droplets using a high voltage. When the solvent has evaporated from the droplets, repulsion disperses the drops into smaller ones. The process is repeated until single ions or adducts are formed.	The eluent is vaporized using a heater, and the ionisation takes place in the gas phase. A corona discharge needle ionizes the analytes by proton transfer or charge exchange reactions.	The ionisation takes place in the gas phase, where the ionisation process is initiated by 10 eV photons. The ionisation of analytes takes place by proton transfer or by charge exchange.
Ideal analytes in general	Polar and ionic compounds, both labile and stabile compounds, eg. proteins, peptides, many of biomolecules	Polar and medium polar, stabile compounds eg.	Polar and non-polar, stabile compounds, eg. PAHs and many lipids.
Ideal steroid analytes	Steroids with the conjugated double-bond structure, all conjugates, bile acids.	Steroids with conjugated double-bond structure or keto group.	Steroids with the conjugated double-bond structure, steroids with low polarity as most of pregnanes.
Non-optimal steroid analytes	Less polar or saturated steroids, as most of pregnanes and DHEA	All conjugates, some gluco- and mineralocorticoids.	All conjugates, some of gluco- and mineralocorticoids.

Analysis of steroids during pregnancy. The analysis of steroids in plasma or urine samples is easier than in brain samples, since urine and plasma samples are not as complex as brain tissue samples and the concentrations are higher, especially in pregnancy samples. Radioimmunoassay (RIA), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography- mass spectrometry (LC-MS) have been commonly used in analysis of steroids in pregnancy samples. RIA is applied for analysis of PROG, AP, and 5α-THDOC during pregnancy [Paoletti et al., 2006; Luisi et al., 2008]. However, the suitability of RIA in the analysis of complicated biological samples, such as pregnancy urine, is questionable due to possible cross reactions. GC-MS providing a more specific method than RIA was applied successfully to the analysis of steroids during pregnancy [Hill et al., 2010a; 2010b; Evans et al., 2005]. Evans et al. showed that five metabolites of PROG increased significantly from 10 to 36 weeks of pregnancy. Glucuronide and sulphate conjugates of steroids have also been analysed by GC-MS after hydrolysis. Meng et al. studied progesterone metabolites and their sulphate and glucuronide conjugates in serum and in pregnancy urine. The conjugates were separated using an ion-exchanger cartridge followed by hydrolysis and analysis by GC-MS, and serum and urine samples from patients with intrahepatic cholestasis of pregnancy (ICP) and those with normal pregnancies (one sample from 31-38 week of gestation) were compared [Meng et al. 1997a; 1997b]. Recently Hill et al. analysed 40 steroids and 26 conjugates in blood samples after chemical hydrolysis by GC-MS using four data points during the pregnancy [Hill et al., 2010]. They observed that the concentrations of conjugated steroids were 5-100 times higher than for non-conjugated steroids in the samples collected at the 3th semester and suggested that the balances between free steroids and their conjugates may be crucial for the regulation of their biological activity and consequently for the stability of pregnancy. LOQs of ng/mL level have been reported for intact steroid glucuronide conjugates in biofluids (Table 5), but they have not earlier measured during pregnancy.

Table 5. *LC-MS analysis of endogenous steroid conjugates from human and animal samples without hydrolysis.*

Technique	Method Validation	Androgen Glu	Estrogen Glu	Corticoid Glu	Steroid Sult	LOD or LOQ ng/mL	Matrix	References
LC-MS/MS	Partly	-	10 Glu	-	No	Not reported	Serum	Caron et al., 2009
LC-MS/MS	Yes	-	-	12 Glu	No	LOQ 2.5-20	Urine	Ikewaga et al., 2009
LC-MS/MS	No	A, TES, epiTES, EtiO	-	-	No	LOD 10	Urine	Pozo et al., 2008
LC-MS/MS	No	A, TES, DHEA	E2	HC	Yes	Not reported	Urine	Antignac et al., 2005
LC-MS/MS	No	A, DHEA, TES, epiTES, EtiO	-	-	No	Not reported	Urine	Biuarelly et al., 2004
LC-MS/MS	Yes	TES, DHT	-	-	No	LOQ 1-10	Urine	Choi et al., 2003
LC-MS/MS	Yes	-	E3	-	No	LOQ 5-10	Urine	Yang et al., 2003
LC-MS/MS	No	DHEA, AN, EtiO	-	-	Yes	Not reported	Urine	Jia et al., 2001
LC-MS/MS	No	TES, epiTES	-	-	Yes	Not reported	Urine	Bowers et al., 1996
LC-FL	No	-	E3	-	No	LOD 14-17	Urine	Iwata et al., 1997
LC-ECD	No	-	2-OHE1, 4-OHE1	-	No	Not reported	Urine	Shimada et al., 1987

Abbreviations: 2-OHE1, 2-hydroxyestrone; 4-OHE1, 4-hydroxyestrone; A, 5β-androstane; EtiO, etiocholanolone; EpiTES, epitestosterone; FL, fluorescence detection, ECD, electrochemical detection; others see chapters abbreviations and abbreviations and structures of steroids and steroid conjugates.

2 Aims of the study

The objective of the study was to develop sensitive LC-MS/MS methods for the analysis of endogenous steroids and their conjugate metabolites from biological matrices. Because the commercial availability of steroid glucuronides is limited, the first objective was to develop a method for the production of reference materials for steroid glucuronides using enzymatic synthesis, and to purify and characterize the material compounds [I]. The second goal was to develop a sensitive and selective analytical method including sample preparation, for reliable determination of steroid and their conjugates in brain tissue extracts [II, III]. The ultimate goal was to discover neurosteroid glucuronides in the rat and mouse brain. The other aim was to apply the developed method to the screening of urine samples during pregnancy [IV].

3 Enzyme-assisted Synthesis and Characterisation of Glucuronide Conjugates of Neuroactive Steroids

Synthesis of standard substances for reference material purposes is needed for the determination of the presence and function of steroid glucuronides in the brain or other tissues, because commercial sources of steroid glucuronide standards are limited or unavailable. In the present study porcine, rat, and bovine liver microsomes were tested to evaluate their ability to glucuronidate eight neurosteroids and neuroactive steroids of various types: dehydroepiandrosterone, pregnenolone, isopregnanolone, 5α-tetrahydrodeoxy-corticosterone, corticosterone, cortisol, β-estradiol, and testosterone. In general, the glucuronidation efficiency of rat liver was rather poor compared with that of bovine and porcine liver microsomes. Since porcine liver apparently has a relatively large amount of dehydrogenase, its microsomes also produced dehydrogenated steroids and their glucuronides, as well as various regional isomers in which the site of glucuronidation varied. In contrast, bovine liver microsomes produced mainly a single major glucuronidation product and few dehydrogenation products and gave the best overall yield for two-third of the steroids tested. The enzymatic synthesis of five steroid glucuronides was carried out and the conditions, purification, and analytical methods for the glucuronidation products were optimized. The steroid glucuronides synthesized were characterized by nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography-mass spectrometry (LC-MS). The stereochemically and structurally pure steroid glucuronide conjugates were recovered in milligram amounts (yield 10-78%) and good purity (>85-90%), which is sufficient for LC-MS/MS method development and analyses of steroid glucuronides in biological matrices such as brain, urine, or plasma.

3.1 Introduction

Brain steroids are usually divided in to two subclasses: neurosteroids and neuroactive peripheral steroids. Neurosteroids differ from peripheral steroids in the way they can be synthesized *de novo* from cholesterol in the central nervous system, in part independently of peripheral sources *[1]*. The brain is considered an important target site of those steroids that regulate several important brain neuronal functions. The pharmacological significance of neurosteroids has been described in several reviews and articles, in which they were involved in the regulation of behavioral and cognitive functions such as memory [*2,3*], stress response [*2,4*], insomnia and sleep [*3,5,6*], and diseases such as anxiety, depression and aggression [*2-5*], attention deficit hyperactivity disorder [*3*], drug dependence [5], epilepsy [*5,7*], schizophrenia and bipolar disorder [*3,4,8*], and Alzheimer's [*9,10*] and Parkinson's diseases [*11*]. At the biochemical level, neuroactive steroids are involved in the modulation of gamma-amino-n-butyric acid A agonist ($GABA_A$), N-methyl-d-aspartate (NMDA), nicotinic acetylcholine, muscarinic, serotonin ($5\text{-}HT_3$), kainate, glycine, and sigma receptors [*2,3,6,12,13*], and induction of neurite outgrowth, dendritic spines, and synaptogenesis [*2,12*]. Although the homeostatic regulation of neurosteroids is not fully understood, neurosteroids are promising neuromodulators that either activate or inactivate neuron-neuron communication and thereby mediate many brain functions [*14*].

Phase I metabolism and biosynthesis of neurosteroids are well documented [*2,15,16*]. Phase II metabolism includes formation of glucuronide and sulfate conjugates, in which steroids are inactivated. Glucuronidation is catalyzed by uridine 5'-diphospo-glucuronosyltransferase (UGTs), which are membrane-bound enzymes of the endoplasmic reticulum, and is the main pathway of conjugation and inactivation reactions of steroids. Most UGTs are expressed in the liver, which is considered to be the major site of glucuronidation. However, some UGTs are extrahepatic enzymes, and many of the UGTs are also found in other tissues [*17*]. UGTs 1A6 [*18,19*], 2A1 [*20*], 2B7 [*18*] and 2B19 [*21*] have been found in brain. In addition, sulphotransferases, enzymes that catalyze sulfate conjugation, exist in the brain [*22-24*], and pregnenolone and dehydroepiandrosterone sulfates were found in the brain, using indirect methods [*25,26*].

Synthesis of material substances for method development and reference material purposes is needed to determine the presence and function of steroid glucuronides in the brain or other tissues. Commercial sources of steroid glucuronide standards are very limited or nonexistent. Chemical syntheses of steroid glucuronides have been reported [*27-32*], but the formation of racemic mixtures of α/β-anomers and by-products is a possible problem of these methods. Induced rat liver microsomes are also used for glucuronidation of steroids [*33-35*] but current research in this field is mainly focused on androgenic anabolic steroids, and enzymatic synthesis methods for other endogenous steroids, e.g. neurosteroids, are still lacking. Induced rat liver microsomes are traditionally used as sources of UGTs, and the use of liver microsomes from other species needs to be evaluated. The glucuronidation efficiency of rat liver microsomes is in some cases too low, especially for reference material production. In the present study porcine, rat and bovine liver microsomes were tested to evaluate their ability to glucuronidate eight neurosteroids or neuroactive steroids of various types by method previously reported with rat liver microsomes [*33*]. In addition, the enzymatic synthesis of five steroid glucuronides was carried out, and the glucuronide conjugates produced were characterized with capillary liquid chromatography-mass spectrometry (CapLC-MS), high resolution mass spectrometry (HRMS), and nuclear magnetic resonance spectroscopy (^{1}H NMR).

3.2 Experimental

Substrates and reagents. Cortisol, dehydroepiandrosterone (DHEA), estradiol, isopregnanolone, pregnenolone (PREG), 5α-tetrahydrodeoxycorticosterone (5α-THDOC), and testosterone were supplied from Sigma-Aldrich (St. Louis, MO, USA), and corticosterone was supplied from Fluka Chemie GmbH (Buchs, Switzerland). Testosterone glucuronide (TG), pregnanolone glucuronide (PG) and dehydroepiandrosterone glucuronide (DG) from Steraloids Inc. (Newport, RI, USA), and estradiol-3-glucuronide (3-EG) from Sigma-Aldrich were used as standards in the interspecies comparison of microsomes. Uridine-5'-diphosphoglucuronic acid (UDPGA, trisodium salt) and saccharic acid-1,4-lactone were purchased from Sigma-Aldrich. Aroclor 1254 (RCS-088/Analabs) was obtained from the Foxboro Company (North Haven, CT, USA). HPLC grade solvents and analytical grade reagents were used in the study.

Liver microsomes. Porcine, bovine, and rat microsomes were isolated from livers with differential centrifugation (*36*), and the protein concentrations of the microsomes were determined with a commercial BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). The porcine and bovine livers were obtained from a commercial slaughterhouse (Paimion teurastamo, Paimio, Finland). The protein activity was 8.38 or 10.29 mg/mL (two batches used) for the porcine liver microsomes and 8.78 mg/mL for bovine liver microsomes. Male Wistar rats treated with Aroclor 1254 (500 mg/5 mL olive oil per kg intraperitoneally 5 days before slaughter) were taken from our previous study [*36*]. The rat liver microsomes were used as a pool of six animals and their protein activity was 15.72 mg/mL. Preparation of the rat liver microsomes was approved by the local Ethical Committee of the Department of Occupational Health (Helsinki, Finland). The microsomes were stored at -70 °C before use.

Interspecies comparison. The synthesis for interspecies comparison was carried out in a total volume of 250 µL containing 10 mM $MgCl_2$, 5 mM saccharic acid-1,4-lactone, and 50 mM phosphate buffer, pH 7.4. Steroid aglycones in dimethyl sulfoxide (DMSO) were added to a final concentration of 0.2 mM in the incubation mixtures. The concentration of DMSO in the reaction solution was 10% (v/v). The protein concentration was 1.0 mg/mL based on a previous study [*33*]. The reactions were initiated by the addition of UDPGA to a final concentration of 5 mM and carried out at 37 °C for at least 12 h. The reactions were terminated by the addition of 25 µL of methanol, and the solutions were transferred to the ice bath. The mixtures were centrifuged for 15 min, the supernatant collected, and the proteins resuspended in 250 µL of 50% methanol and further centrifuged. The supernatants were combined and subjected to a CapLC-MS analysis.

Biosynthesis. The synthesis of isopregnanolone glucuronide (IPG), 5α-THDOC 3- and 21-glucuronides (3- and 21-THDG), corticosterone 21-glucuronide (21-CG), and cortisol 21-glucuronide (21-HCG) was carried out in a total volume of 40 mL, containing the same concentrations of reagents and proteins as in the small-scale synthesis. Steroid aglycones in DMSO (10%, v/v) were added to a final concentration of 0.2 mM (isopregnanolone and 5α-THDOC) or 0.5 mM (corticosterone and cortisol) in the reaction mixtures. The reactions were carried out at 37 °C for 24 h, and terminated by the addition of 4 mL of 4 M perchloric acid (IPG) or methanol (THDGs, 21-CG, 21-HCG), and the solutions were transferred to the ice bath. The mixtures were centrifuged for 15 min, the supernatant collected, and the precipitated proteins resuspended with 5 mL of 50% methanol and recentrifuged. The supernatants were combined and subjected to purification.

Purification of bulk samples. The supernatants containing the steroid glucuronides were purified by solid-phase extraction (SPE) using Oasis HLB cartridges (6 mg, 3 cc, Waters, Milford, MA, USA). The cartridges were preconditioned with 3 mL of methanol and water. After loading a 10-mL quantity of the sample, the cartridges were rinsed either with water (21-HCG), 10% methanol (21-CG), or 20% methanol (IPG, THDGs). The 21-HCG sample was diluted with 10 mL of water prior to purification. The glucuronides were eluted with 3 mL of the extraction solvent, which was selected so that aglycone was retained in the cartridge at the same time as the glucuronide was eluted. The extraction solvents selected were 65% and 60% methanol for IPG and THDGs, respectively, and 40% and 25% acetonitrile for 21-CG and 21-HCG, respectively. The organic fraction of the extract was evaporated at 50 °C under nitrogen, and the remaining extract (water phase) was frozen and lyophilized. Regional isomers and a dehydrogenation glucuronide impurity in the THDG were separated by HPLC fraction collection. The HPLC fraction collection setup consisted of an

Agilent 1100 binary pump equipped with an auto sampler and a fraction collector. The column used was a Hypersil BDS-C18 250 x 4.0 mm, 5 µm (Agilent, Palo Alto, CA, USA), the flow rate was 1 mL/min, the injection volume was 100 µl, and the gradient used was 0 min 5% B, 1 min 40% B, 7 min 42% B, 12 min 45% B, 13-14 min 100% B, 15 min 5% B, in which A was 50 mM ammonium acetate at pH 4.5 (adjusted by acetic acid):water (1:9 v/v) and B was 50 mM ammonium acetate at pH 4.5 (adjusted with acetic acid) :methanol: acetonitrile (1:3:6 v/v). The equilibrium time between runs was 15 min. UV detection with a wavelength of 210 nm was used, and the fractions were collected at retention times of 7.3-7.9 min (3-THDG) and of 9.5-10.7 min (21-THDG). The salts were removed with SPE, using the same conditions as with the supernatants in bulk synthesis, except that the elution was made with methanol: acetonitrile (1:1 v/v), and the samples were evaporated at 50 °C under nitrogen. The samples were dried in vacuum, weighed, and subjected to analysis.

Liquid chromatography-mass spectrometry. The HPLC consisted of a Agilent 1100 binary pump, an Agilent 1100 Capillary LC system equipped with an auto sampler and 100-µL injection loop (Agilent, Waldbronn, Germany), and the mass spectrometer used was an API 3000 triple quadrupole instrument (MDS Sciex, Toronto, ON, Canada) with a turbo ion spray source. The analyses were carried out using two end-capped columns (Symmetry Shield RP-18, 50 x 1 mm, 3.5 µm and 100 x 0.3 mm 3.5 µm, Waters, MA, USA). The temperature of the columns was 50 ˚C, the injection volume was 1-10 µL, the gradient program was 0 min 5% B, 0.1 min 45% B, 7 min 75% B, 10 min 85% B, and 11-25 min 100% B, and the equilibrium time between runs was 20 min. The eluents were the same as in the fraction collection method. A flow rate of 20 µL/min was used for the washing period (0-7min, 25-27 min) and 9 µL/min for the separation period (7-25 min). During the washing period the mobile phase was directed from column 1 to waste, while the second pump was simultaneously used to balance the analytical column (column 2). During the separation period the analytes were eluted from column 1 to the analytical column and later to the mass spectrometer. The ion spray voltage was 5500 V in the positive ion mode and -4200 V in the negative mode, and the scanning range was from *m/z* 50 to 600, and the scanning rate was one spectrum in 2 s. The orifice voltage and collision energy were 30 V and 30 V in the positive mode and -60 V and -60 V in the negative mode, respectively. Purified air (Atlas Copco, Overijse, Belgium) was used as a nebulizing gas and nitrogen (Generator 75-72, Whatman, Clifton, NJ) as the curtain, collision, and turbo gases. The data were processed with Analyst 1.4. software (Applied Biosystems/MDS Sciex).

High resolution mass spectrometry. Accurate mass measurements were performed with a quadrupole-time-of-flight (Q-TOF Micro) instrument, (Waters Micromass, Manchester, UK) with electrospray ionisation and the data were processed with Masslynx 4.0 software (Waters Micromass). Injection of the sample was done by Agilent 1100 HPLC auto sampler. The isocratic eluents were 50 mM ammonium acetate, pH 4.5 (acetic acid) as A (40%) and methanol as B (60%), and the flow rate of the mobile phase was 0.1 mL/min. The capillary voltage was 2000 V and the sample cone voltage 23 V. The temperatures of the source and desolvation gas were 100 ˚C and 300 ˚C, respectively. Nitrogen (Generator BN15L; Peak Scientific, Bedford, MA, USA) was used as the desolvation gas and the flow was set up to 600 L/h, cone gas was not used. A sodium formate solution was used in calibration of the mass axis, and corticosterone (m/z 347.2222) was used as a lock mass compound. The resolution of the mass spectrometer was 5100-5500. Control samples (testosterone) were

analyzed at the beginning and end of the sample series to check the mass accuracy of the instrument (the specification was 5 ppm).

NMR Spectrometry. The NMR samples were dried in high vacuum and dissolved in 600 µL of deuterated methanol (CD_3OD). The spectra from steroid glucuronides produced were compared with those of the respective steroids. The NMR experiments were carried out on a Varian Mercury Plus 300 spectrometer (Varian, Palo Alto, CA, USA) at 23 ºC. The ^{1}H chemical shifts were referenced to tetramethylsilane (TMS) signal (0 ppm).

3.2 Results and discussion

Interspecies differences of liver microsomes. Rat, porcine, and bovine liver microsomes were tested as sources for the enzymatic synthesis of neurosteroid glucuronides. The glucuronides produced in 12 h incubations were identified, using CapLC-MS and commercial reference materials (3-EG, TG, DG, PG) or standards produced in this study (IPG, 21-CG, 21-HCG, 3- and 21-THDG). The results in Table 3.1 clearly show that good or moderate glucuronidation efficiency was recognized for all the steroids, using either porcine or bovine liver microsomes, whereas only 5α-THDOC, testosterone and estradiol were glucuronidated efficiently with rat microsomes.

Those steroids having more than one glucuronidation site produced regional isomers of monoglucuronides, which were at least baseline-separated by the CapLC-MS method. The 21-glucuronide was detected as the main product in all glucocorticoids (corticosterone, cortisol, 5α-THDOC), but the glucuronidation of 11- and 17β-OH was inefficient, most probably due to steric hindrance by neighboring rings and a carbonyl side chain. The same type of steric hindrance was reported with methyl testosterone in previous study, in which 17α-CH_3 substitution inhibited 17β-OH glucuronidation [*37-38*]. The steric hindrance of 17β-OH was also probably the reason for low yield of 21-glucuronide of cortisol. Both possible glucuronidation sites of 5α-THDOC, 21- and 3α-OH were efficiently glucuronidated with all the microsomes. In the case of estradiol, phenolic hydroxyl at the 3-position was efficiently glucuronidated with all microsomes, whereas 17-OH was glucuronidated only with rat liver microsomes. In general, the rat and porcine liver microsomes produced higher amounts of the regional isomers than the bovine liver microsomes.

Significant amounts of dehydrogenation product glucuronides were produced in porcine liver microsomes, using the glucocorticoids (Table 3.1). The dehydrogenation products (e.g. 11-keto metabolites) are known metabolites of glucocorticoids [*39*] and were formed in our experiments due to traces of CYP enzymes and $NADP^+$ in porcine microsomes. Furthermore, several minor phase I metabolites (consecutive hydrogenation and dehydrogenation products) were detected for both corticosterone and cortisol by the porcine liver microsomes. Since the porcine and bovine liver microsomes glucuronidated neurosteroids more efficiently than the rat liver microsomes and the bovine liver microsomes produced fewer regional isomers and dehydrogenation products than the porcine liver microsomes, the bovine liver microsomes were preferred in enzymatic synthesis of neurosteroid glucuronides.

Table 3.1. *Comparison of relative glucuronidation efficiency of and amounts of dehydrogenation side products by rat, porcine and bovine liver microsomes. Since the response factors of the regional isomers or impurities are not the same as those of the main compound, only the relative differences of results can be compared. The recovery of the main products is obtained by comparison of the peaks of the external glucuronide standards or from the absolute yields of synthesis.*

Substrate	Structure of substrate	Main products and impurities	Relative glucuronidation efficiency			Recovery of main products by the most effective species
			Rat liver [1]	Porcine liver	Bovine liver	
Iso-pregnanolone		3-IPG	0	100	78	+++
PREG		3-PG	0	86	100	+++
Corticosterone		21-CG 21-CG: 11-CG: dehydrogenation CG	4 100:0:5	56 100:1:73	100 100: 0.3:2	++
Cortisol		21-HCG 21-HCG: 11-HCG[2]: dehydrogenation HCG	8 100:0:33	15 100:14:28	100 100:4:2	+
5α-THDOC		3-THDG 21-THDG 3-THDG: 21-THDG: dehydrogenation THDG	33 46 100:82:8	30 100 55:100:9	100 62 100:34:8	+++ +++
Testosterone		17-TG	100	86	52	++
DHEA		3-DG	4	100	99	+++
Estradiol		3-EG 3-EG:17-EG	50 100:17	90 100:0	100 100:0	+++

+++ good, ++ moderate, + poor. [1] Rat liver microsomes were induced with Aroclor 1254. [2] Only one regional isomer was observed as an impurity. Glucuronidation site of 17-cortisol glucuronide is stericly hindered by ring structure and neighboring carbonyl side chain and is not likely produced.

Biosynthesis. The synthesis was performed in a reaction volume of 40 mL using 8 µmol (isopregnanolone, 5α-THDOC) or 20 µmol (corticosterone, cortisol) of steroid aglycone to produce glucuronide standards in milligram quantities. The solubility of the lipophilic substrates in the aqueous incubation media used is poor, and therefore organic solvent was added to the reaction mixture. The concentration of DMSO was limited to 10% to avoid meaningful decrease in enzyme activity and the concentration of the substrates was kept low, below 0.2 – 0.5 M.

After synthesis the supernatant was collected and the microsomal fraction was resuspended in 50% methanol to improve the total yield. For example, 50% of more lipophilic IPG (log P 2.9) and 7% of the less lipophilic 21-CG (log P 1.8) were found in resuspended fraction. The results clearly show that the steroid glucuronides are significantly bound by microsomal proteins and that the binding is dependent on the lipophilicity of the steroid glucuronide. We also observed that steroid glucuronides are sensitive to acidic hydrolysis, and therefore the reaction termination step had to be performed rapidly at low temperature when perchloric acid was used. Termination at room temperature significantly decreased the yields for glucocorticoid glucuronides, and hydrolysis can be avoided by using organic solvent in the termination. With 21-CG and 21-HCG, 1.3 and 1.5 times better recoveries were obtained, when the reaction was terminated by methanol instead of perchloric acid.

We minimized the number of purification steps to speed up the purification process and to obtain as high recoveries as possible. The purification of IPG, 21-CG, and 21-HCG from starting materials and minor impurities by SPE was sufficient. 5α-THDOC produced two glucuronide regional isomers (21-THDG and 3-THDG) and dehydrogenated THDG. In this case HPLC separation with fraction collection was needed for the purification. The synthesis products were fully separated with the HPLC method used. The retention times for 3-THDG, dehydrogenated THDG and 21-THDG were 7.6 min, 8.1 min, and 10.1 min, respectively. Since the synthesized glucuronides have poor UV absorbance, the purity of the isolated compounds was measured in ^{1}H NMR and CapLC-MS analyses. The purities were better than 85-90% (Table 3.2) being sufficient for the use of reference compounds in detection and analysis of the glucuronides in brain samples. After purification the yields were 0.4-3.1 mg (10-78 %) (Table 3.2), which was sufficient for accurate weighing and for structural characterization by NMR and MS.

In conclusion, the optimized methods were used to synthesize and purify five glucuronides of four neuroactive steroids. In most cases, SPE was sufficient to achieve adequate purification of the steroid glucuronides. In cases in which several glucuronidation sites (e.g. hydroxyl groups) were available, thus producing several regional isomers or dehydrogenated steroids and their glucuronide conjugates, fraction collection with HPLC was needed to separate these compounds of similar lipophilicity. The method up scaling is possible simply using larger incubation volumes to produce tens or hundreds milligram amounts of steroid glucuronides.

Table 3.2. *Summary of neurosteroid glucuronides synthesized and characterized in the study.*

Abbreviation	*Precursor*	*Site of glucuronidation*	*Source of microsomes*	*Yield*		*Purity*[a]
				mg	%	%
IPG	Isopregnanolone	3β-	Porcine	3.1	78	> 90
3-THDG	5α-tetrahydro-deoxycorticosterone	3α-	Bovine	2.8	69	> 90
21-THDG	5α-tetrahydro-deoxycorticosterone	21-	Bovine	0.5	12	> 90
21-CG	Corticosterone	21-	Bovine	2.5	60	> 85
21-HCG	Cortisol	21-	Bovine	0.4	10	> 90

[a] Estimation by ^{1}H- NMR and CapLC-MS analyses

Characterization. The mass spectra of the synthesized steroid glucuronides were measured, using CapLC-ESI/MS both in the positive and negative ion modes. We detected ammonium adduct ions $[M+NH_4]^+$ and protonated molecules $[M+H]^+$ in the positive ion mode and deprotonated molecules $[M-H]^-$ in the negative mode indicating the correct molecular weights of the synthesized glucuronides (Table 3.3). Ammonium adducts predominated in the mass spectra and protonated molecules were formed only with 21-CG and 21-HCG. The conjugated carbonyl group in 21-CG and 21-HCG has high proton affinity, thus enabling the proton transfer reaction. The proton affinities of IPG, 3-THDG, and 21-THDG are too low for protonation but sufficiently high for formation of ammonium adducts. The product ion spectra (Table 3.3) show the diagnostic and abundant ions formed by neutral loss of the glucuronide moiety (176 amu) ($[M+H-GLU]^+$), with subsequent loss of one or two water molecules ($[M+H-GLU-nH_2O]^+$) in the positive ion mode and $[M-H-GLU-H_2O]^-$ in the negative ion mode. The product ions at m/z 177 ($[GLU+H]^+$), m/z 159 ($[GLU+H-H_2O]^+$), m/z 141 ($[GLU+H-2H_2O]^+$), and m/z 113 ($[GLU+H-2H_2O-CO]^+$) in the positive mode, and at m/z 175 ($[GLU-H]^-$), m/z 113 ($[GLU-H_2O-CO_2]^-$), and m/z 85 ($[GLU-H-CH_2OHCH_2COOH]^-$) in the negative mode, provided additional evidence for glucuronidation of the substrates. The two regional isomers 3- and 21-THDG can be separated by the intensity ratio of $[M+H-GLU]^+/[GLU+H]^+$, which is significantly greater with 21-THDG than with 3-THDG.

HRMS was used to determine the elemental composition of the products (Table 3.4). The measured accurate masses of $[M+H]^+$, $[M+NH_4]^+$, and the fragment ions $[M+H-GLU]^+$ and $[M+H-GLU-H_2O]^+$ produced by in-source dissociation correspond well with the calculated exact masses indicating the correct elemental composition of all the synthesized steroid glucuronides. The relative errors of all measured masses were below 4.7 ppm.

The ^{1}H NMR spectra of each steroid glucuronide were assigned for structural confirmation. The anomericity of the glucuronide moieties was determined from the vicinal coupling constant $^{3}J_{H1-H2}$. Since the coupling constants determined (7.2-7.8 Hz) are typical values between two axial protons, the *β*-configuration was confirmed. Peak integration of each steroid glucuronide spectrum showed a 1:1 relationship between the aglycone and glucuronic acid moieties. Glucuronidation occurred at the 3-position in isopregnanolone and

was shown by the presence of abroad multiplet of aglycone H3 at 3.75 ppm. In the THDG, the 3- and 20-positions were glucuronidated. Shifting of steroid H3 (3.95→3.97 ppm) indicated glucuronidation in the 3-position. On the other hand, shifting and splitting of two geminal protons in aglycone H19 (4.66 and 4.31 ppm, d, J = 17.7 Hz) establish glucuronidation of the hydroxyl group at the 20-position. Another indicator of attachment at the 20-position was a shifting of methine proton H17 on the D-ring (2.58→2.68 ppm). With corticosterone, glucuronidation occurred at the 20-position rather than at the 11-position of the secondary hydroxyl. Geminal methylene protons of H19 were shifted and split (4.54 and 4.30 ppm, d, J = 18.0 Hz) as was methine proton H17 on the D-ring (2.52→2.68 ppm). Cortisol was also glucuronidated at the hydroxyl on the 20-position, which was indicated by a large shift of geminal methylene protons of H19 (5.0 and 4.47 ppm, d, J = 18.3 Hz).

Table 3.3. *Characterization of main products by CapLC-MS and -MS/MS.*

Ion	***Product/ m/z*** (relative abundance)				
Positive and Negative Ion CapLC-MS	**IPG**	**3-THDG**	**21-THDG**	**21-CG**	**21-HCG**
$[M+NH4]^+$	512 (100)	528 (100)	528 (100)	540 (100)	556 (29)
$[M+H]^+$	495 (0)	511 (0)	511 (0)	523 (71)	539 (100)
$[M-H]^-$	493(100)	509(100)	509(100)	521(100)	538 (100)
Positive Ion CapLC-MS/MS					
Precursor	512	528	528	540	539
$[M+NH4]^+$	512 (13)	n.d.	n.d.	n.d.	n.d.
$[M+H]^+$	n.d.	n.d.	n.d.	523 (92)	539 (<1)
$[M+H-GLU]^+$	n.d.	335 (32)	335 (95)	347 (100)	363 (77)
$[M+H-GLU-H_2O]^+$	301 (100)	317 (100)	317 (100)	329 (92)	345 (100)
$[M+H-GLU-2H_2O]^+$	283 (88)	299 (68)	299 (61)	311 (28)	327 (50)
$[M+H-GLU-3H_2O]^+$	n.d.	n.d.	n.d.	293 (17)	309 (27)
$[M+H-GLU-4H_2O]^+$	n.d.	n.d.	n.d.	n.d.	291 (9)
Other [a]	n.d.	n.d.	n.d.	n.d.	297 (24)
$[GLU+H]^+$	177 (8)	177 (24)	177 (2)	177(1)	n.d.
$[GLU+H-H_2O]^+$	159 (8)	159 (26)	159 (14)	159 (11)	159 (9)
$[GLU+H-2H_2O]^+$	141 (35)	141 (46)	141 (6)	141 (4)	141 (8)
$[GLU+H-H_2O-CO]^+$	n.d.	131 (2)	131 (2)	131 (3)	131 (2)
$[GLU+H-2H_2O-CO]^+$	n.d.	113 (2)	113 (4)	113 (3)	113 (3)

Ion	**Product/ m/z** (relative abundance)				
Negative Ion CapLC-MS/MS					
Precursor	493	509	509	521	537
$[M-H]^-$	493 (0)	509 (0)	509 (1)	521 (2)	537 (3)
$[M-H-GLU]^-$	317 (3)	333 (4)	333 (32)	345 (9)	n.d
$[M-H-GLU-H_2]^-$	315 (8)	331 (5)	331 (3)	343 (2)	n.d
$[M-H-GLU-H_2-CO]^-$	n.d	303 (2)	303 (4)	315 (2)	331 (22)
$[M-H-GLU-H_2O]^-$	299(6)	315 (2)	315 (2)	327 (19)	343 (11)
Other[a]	n.d.	n.d.	n.d.	241 (49),	297 (47), 282 (35),
			.	189 (31)	189 (21)
$[GLU-H]^-$	n.d.	n.d.	n.d.	175 (11)	175 (6)
$[GLU-H-CO_2]^-$	n.d.	n.d.	131 (2)	131 (8)	131 (6)
$[GLU-H-H_2O-CO]^-$	129 (3)	129 (3)	129 (2)	129 (5)	129 (4)
$[GLU-H-2CO-H_2]^-$	117 (4)	117 (6)	117 (6)	117 (15)	n.d.
$[GLU-H-CH_3COOH]^-$	115 (3)	115 (3)	115 (6)	115 (12)	115 (11)
$[GLU-H-CO_2-H_2O]^-$	113 (21)	113 (28)	113 (20)	113 (69)	113 (6)
$[GLU-H-2CO-H_2-H_2O]^-$	99 (12)	99 (7)	99 (7)	99 (26)	99 (22)
$[GLU-H-CH_3COOH-H_2O]^-$	97 (5)	n.d.	97 (6)	97 (13)	97 (2)
$[GLU-H-CO_2-2H_2O]^-$	95 (13)	95 (16)	95 (4)	95 (10)	95 (10)
$[GLU-H-CH_3COOH-CO]^-$	87 (8)	87 (13)	87 (9)	87 (35)	87 (56)
$[GLU-H-CH_2OHCH_2COOH]^-$	85 (100)	85 (100)	85 (100)	85 (100)	85 (100)
$[CH_2OHCOO]^-$	75 (74)	75 (89)	75 (57)	75 (92)	75 (67)
$[CH_3CH_2COO]^-$	73 (11)	73 (12)	73 (7)	73 (31)	73 (23)
$[GLU-H-CH_3COOH-CO_2]^-$	71 (12)	71 (11)	71 (10)	71 (25)	71 (45)

n.d.= not detected [a] relative abundance > 20 %

Table 3.4. *Characterization of synthesized steroid glucuronides by Q-TOF accurate mass measurements. The accurate masses were measured for the ammonium adducts or protonated molecules and for a single fragment ion.*

Compound	Measured mass	Calculated mass	Origin of ion	Molecular formula	Relative error, ppm
IPG	512.3247	512.3223	$[M+NH_4]^+$	$C_{27}H_{46}O_8 N_1$	4.6
	301.2536	301.2531	$[M+H\text{-}GLU\text{-}H_2O]^+$	$C_{21}H_{33}O_1$	1.5
3-THDG	528.3165	528.3173	$[M+NH_4]^+$	$C_{27}H_{46}O_9N_1$	2.3
	335.2583	317.2481	$[M+H\text{-}GLU\text{-}H_2O]^+$	$C_{21}H_{35}O_3$	0.1
21-THDG	528.3165	528.3173	$[M+NH_4]^+$	$C_{27}H_{46}O_9N_1$	2.0
	335.2583	335.2586	$[M+H\text{-}GLU]^+$	$C_{21}H_{35}O_3$	-4.0
21-CG	523.2543	523.2543	$[M+H]^+$	$C_{27}H_{39}O_{10}$	3.9
	347.2207	347.2222	$[M+H\text{-}GLU]^+$	$C_{21}H_{31}O_4$	4.4
21-HCG	539.2513	539.2492	$[M+H]^+$	$C_{27}H_{39}O_{11}$	3.9
	363.2178	363.2171	$[M+H\text{-}GLU]^+$	$C_{21}H_{31}O_5$	1.9

As a conclusion, the analyses with CapLC-MS and -MS/MS in combination with accurate mass measurement and ^{1}HNMR reliably determined the structures of the glucuronides produced, and therefore the synthesised steroid glucuronides can be further used for the development of methods for the analysis of neurosteroid glucuronides using LC-MS/MS, and for other research purposes. This procedure developed can also be easily utilized to produce glucuronide conjugates of other types of steroids, e.g. to produce labelled reference materials using deuterated UDPGA or steroids as a starting material. Thus enzyme-assisted synthesis is an efficient and easy way to produce steroid glucuronides using readily available porcine and bovine liver microsomes.

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3.5 References

[1] Baulieu, E. E. (1997) Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog. Horm. Res. 52,* 1-32.
[2] Mellon, S. H., and Griffin, L. D. (2002) Neurosteroids: biochemistry and clinical significance. *Trends Endocrin. Met. 13*, 35-43.
[3] Rupprecht, R. (2003) Neuroactive steroids: mechanism of action and neuropsychopharmacological properties. *Psyconeuroendocrino. 28*, 139-168.
[4] Pisu, M. G., and Serra, M. (2004) Neurosteroids and neuroactive drugs in mental disorders. *Life Sci. 74*, 3181-3197.
[5] Gasior M., Carter, R. B., and Witkin, J. M. (1999) Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders. *Trends Pharmacol. Sci. 20*, 107-112.
[6] Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., and Paul, S. M. (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science 232*, 1004-1007.
[7] Reddy, D. S. (2004) Role of neurosteroids in catamenial epilepsy, *Epilepsy Res. 62*, 99-118.
[8] Marx, C. E., Stevens, R. D., Shampine, L. J., Uzunova, V., Trost, W.T., Butterfield, M. I., Massing, M.W., Hamer, R. M., Morrow, A. L., and Lieberman, J. A. (2005) Neuroactive steroids are altered in schizophrenia and bipolar disorders: relevance to pathophysiology and therapeutics. *Neuropsychopharmcol.* 1-15.
[9] Schumacher, M., Weill-Engerer, S., Liere, P., Robert, F., Franklin, M. R. J., Garcia-Segura, L. M., Lambert, J. J., Mayo, W., Melcangi, R. C, Parducz, A., Suter, U., Carelli, C. , Baulieu, E. E., and Akwa Y. (2003) Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Progr. Neurobiol. 71*, 3-29.
[10] Weill-Engerer, S., David, J-P., Sazdovitch, V., Liere, P., Eyechenne, B., Pianos, A., Schumacher, M., Delacourte, A., Baulieu, E. E., and Akwa Y. (2002) Neurosteroid quantification in human brain regions: comparison between Alzheimer's and nondemented patients. *J. Clin. Endocrin. Metab. 87*, 5138-5143.
[11] di Michele, F., Longone, P., Romeo, E., Lucchetti, S., Brus, L., Pierantozzi, M., Bassi, A., Bernardi, G., and Stanzione, P. (2003) Decreased plasma and cerebrospinal fluid content of neuroactive steroids in Parkinson's disease. *Neurol. Sci 24*, 172-173.
[12] Stoffel-Wagner, B. (2003) Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann. NY Acad. Sci. 1007*, 64-78.
[13] Belelli, D., and Lambert, J. L. (2005) Neurosteroid: endogenous regulators of the GABA A receptor. *Nat. Rev. Neurosci.* ***6*****,** 565-575.
[14] Kawato, S., Yamada, M., and Kimoto, T. (2001) Brain neurosteroids are 4th generation neuromessengers in the brain: cell biophysical analysis of steroid signal transduction. *Adv. Biophys. 37,* 1-30.
[15] Stoffel-Wagner, B., (2001) Clinical study: neurosteroid metabolism in the human brain. *Eur. J. Endocriol. 145***,** 669-679.
[16] Mensah-Nyagan, A. G., Do-Rego, J-L., Beaujean, D., Luu-The, V., Pelletier, G., and Vaudry, H. (1999) Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev. 51,* 63-81.

[17] Tukey, R. H., and Strassburg, P. (2000) Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Ann. Rev. Pharmacol. 40*, 581-616.
[18] King, C. D., Rios, G. R., Assouline, J. A., and Tephly, T. R. (1999) Expression of UDP-glucuronosyltransferases (UGTs) 2B7 and 1A6 in the human brain and identification of 5-hydroxytryptamine as a substrate. *Arch. Biochem. Biophys. 365* (1), 156-162.
[19] Suleman, F. G., Abid, A., Gradinaru, D., Daval, J-L., Magdalou, J., and Minn, A. (1998) Identification of the uridine diphosphate glucuronosyltransferase isoform UGT1A6 in rat brain and in primary cultures of neurons and astrocytes. *Arch. Biochem. Biophys 358* (1), 63-67.
[20] Jedlitschky, G., Cassidy, A. J., Sales, M., Pratt, N., and Burchell, B. (1999) Cloning and characterization of a novel human olfactory UDP-glucuronosyltransferase. *Biochem. J. 340,* 837-843.
[21] Bélanger, G., Barbier, O., Hum, D. W., and Bélanger, A. (1999) Molecular cloning, expression and characterization of a monkey steroid UDP-glucuronosyltransferases, UGT2B19, that conjugates testosterone. *Eur. J. Biochem. 260*, 701-708.
[22] Shimizu, M., and Tamura., H. (2002) Identification and localization of two hydroxysteroid sulfotransferases in the brain. *J. Health Sci. 48* (5), 467-472.
[23] Shimizu, M., Nakano, Y., Yamasaki, T., Kimoto, T., Kawato, S., and Tamura, H. (2004) Regio- and isoform-specific expression of hydroxysteroid sulfotransferases in rat brain. *J. Health Sci. 50*(6), 689-692.
[24] Shimada, M., Yoshinari, K., Tanabe, E., Shimakawa, E., Kobashi, M., Nagata, K., and Yamazoe, Y. (2001) Idenfication of ST2A1 as a rat brain neurosteroid sulfotransferase mRNA. *Brain Res. 920***,** 222-225.
[25] Corpéchot, C., Robel, P., Axelson, M., Sjövall, J., and Baulieu, E. E. (1981) Characterization and measurement of dehydroepiandrosterone sulfate in rat brain *Proc. Natl. Acad. Sci. U.S.A. 78***,** 4704-4707.
[26] Corpéchot, C., Synguelakis, M., Talha, S., Axelson, M., Sjövall, J., Vihko, R., Baulieu, E. E., and Robel, P. (1983) Pregnanolone and its sulfate ester in rat brain, *Brain Res. 270***,** 119-125.
[27] Kaspersen, F. M., and Van Boeckel, C. A. (1987) A review of the methods of chemical synthesis of sulfate and glucuronide conjugates. *Xenobiotica 17*, 1451-1471.
[28] Stachulski, A. V., and Jenkins, G. N. (1998) The synthesis of O-glucuronides. *Nat. Prod. Rep. 15*, 173-186.
[29] Conrow, R. B., and Bernstein, S. (1971) Steroid conjugates. VI An improved Koenigs-Knorr synthesis of aryl glucuronides using cadmium carbonate, a new effective catalyst. *J. Org. Chem. 36*, 863-870.
[30] Sanaullah, and Bowers, L. D. (1996) Facile synthesis of [16,16,17-2H3]-testosterone, -epitestosterone and their glucuronides and sulfates. *J. Steroid Biochem. 58*, 225-234.
[31] Chung, B. C., Mallamo, J. P., Juniewicz, P. E., and Shackleton, C. H. L. (1992) Synthesis of deuterium-labelled 5α-androstane-α, 17β-diol and its 17β-glucuronide. *Steroids 57*, 530-536.
(32] Rao, P. N., Rodriquez, A. M., and Miller, D. W. (1986) Synthesis of 5α-androstane-3α, 17β-diol 3- and 17-glucuronides. *J. Steroid Biochem. 25*, 417-421.
[33] Kuuranne, T., Aitio, O. Vahermo, M., Elovaara, E., and Kostiainen, R. (2002) Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of

methyltestosterone (17α-methylandros-4-en-17β-ol-3-one) and nandrolone (estr-4-en-17β-ol-3-one) Metabolites. *Bioconjugate Chem. 13,* 194-199.
[34] Rittmaster, R. S., Leopold, C. A., and Thompson, D. L. (1989) Androgen glucuronyl transferase activity in rat liver, evidence for the importance of hepatic tissue in 5α-reduced androgen metabolism. *J. Steroid Biochem. 33*, 1207-1212.
[35] Numazawa, M., Suzuki, K., and Nambara, T. (1977) Glucuronidation of testosterone by rat liver microsomes. *Chem. Pharm. Bull. 25*, 253-258.
[36] Luukkanen, L., Elovaara, E., Lautala, P., Taskinen, J., and Vainio, H. (1997) Characterization of 1-hydroxypyrene as a novel marker substrate of 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase(s). *Pharmacol. Toxicol. 80*, 152-158.
[37] Schänzer, W. (1996) Metabolism of anabolic androgenic steroids. *Clin. Chem. 42*, 1001-1020.
[38] Kuuranne, T., Kurkela, M., Thevis, M., Schänzer,W., Finel M., and Kostiainen R. (2003) Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab. Dipos.* 31, 1117-1124.
[39] Funder, J. W., Pearce P. T., Smith R., and Smith A. I. (1988) Mineralocorticoid action: target tissue specificity is enzyme not receptor-mediated. *Science 242*, 583-585.

4 Discovery of Neurosteroid Glucuronides in Mouse Brain

Neurosteroid glucuronides were found for the first time in brain samples. The intact glucuronides were extracted from the cortex, hippocampus, hypothalamus, and mid-brain tissues of nicotine- and water-treated mice, and detected with capillary liquid chromatography-electrospray-tandem mass spectrometry (CapLC-ESI-MS/MS). The glucuronides of estradiol, cortisol, corticosterone, tetrahydrodeoxycorticosterone, pregnenolone, and isopregnanolone were identified by comparing retention times in selected reaction monitoring (SRM) chromatograms and the relative abundances of two SRM transitions of each neurosteroid glucuronide between the reference and authentic samples, thus providing reliable identification. *In vitro* experiments, carried out by using S9 fractions from mouse and rat brains, showed a formation of glucuronides with selected test compounds (corticosterone, pregnenolone, and dehydroepiandrosterone), suggesting that biosynthesis of neurosteroid glucuronides is possible in rodent brain.

4.1 Introduction

The role of the metabolism of neurotransmitters and modulators is a key point in our understanding of brain functions. Neurosteroids are an important class of neuromodulators that can either activate or inactivate neuron-neuron communication, thereby mediating many brain functions. Neurosteroids and their sulphate metabolites have been shown to modulate a variety of neurotransmitter systems such as $GABA_A$, cholinergic, opioid, N-methyl-d-aspartate (NMDA), nicotinic, muscarinic, serotonin (5-HT(3)), kainate, glycine and sigma receptors, as well as the induction of neurite outgrowth, dendritic spines, and synaptogenesis [1-3].

The metabolism of neurosteroids has been widely studied, though most such studies have focused on well-known phase I metabolism pathways [4-6]. The role of the phase II metabolism of neurosteroids, however, is less studied and understood. The main phase II conjugation reactions of many steroids are sulphation and glucuronidation catalysed by sulphotransferases (SULT) and glucuronosyltransferases (UGT), respectively. Of these transferases, SULT 2A1 [7-8], SULT 2A2 [9], and SULT 2B1b [7], UGT 1A6 [10-11], UGT 2B7 [10], as well as UGT 2B19 [12] isoenzymes have been found in the rat, human, or monkey brain.

The importance of two neurosteroid sulphates, pregnenolone (PREGS) and dehydroepiandrosterone (DHEAS) sulphates, has been described in several reviews e.g. sulphate conjugates can affect neuronal activity via the modulation of sigma 1 receptors, GABAergic, and glutamatergic neurotransmission [13-14]. Significant amounts of PREGS and DHEAS sulphates have been measured using indirect methods (the sulphates were analysed as their aglycones with gas chromatography-mass spectrometry or radioimmunoassay after enzymatic or acidic hydrolysis) in human brain tissue [15]. Both of sulphate conjugates have also been detected in the rat brain using indirect methods [16-17]. The reliability of these results is questionable, however, since more reliable direct methods, in which the intact sulphates are analysed directly without hydrolysis, have failed to detect neurosteroid sulphates in the rodent brain [18-19].

In contrast to neurosteroid sulphates, no neurosteroid glucuronides have been detected in brain or cerebrospinal fluid thus far, even though glucuronidation is the most common conjugation reaction in the liver, and specific glucuronosyltransferases [10-12] have been found in the brain. In fact, the presence of glucuronides in the rodent brain has been shown only once, as Uutela et al. recently (2009) detected intact dopamine glucuronide in rat brain microdialysates by using LC-MS/MS [20]. Dopamine glucuronide has also been found earlier in the cerebrospinal fluid of the rat using an indirect method [21]. These findings suggest that glucuronidation may play an important role in the metabolism of neurotransmitters.

The aim of this study was to develop a novel direct method for the identification of intact neurosteroid glucuronides in the rodent brain without hydrolysis step by using capillary liquid chromatography-electrospray-tandem mass spectrometry (CapLC-ESI-MS/MS). The method provides more reliable identification of the glucuronides than the previous indirect methods with hydrolysis step and analysis of aglycones instead of intact glucuronides. The second aim was to study the formation of neurosteroid glucuronides in vitro using S9 fractions of mouse and rat brain containing cytosolic and microsomal enzymes.

4.2 Experimental

Reagents and standards. Sigma (St. Louis, MO, USA) supplied Estradiol-3-glucuronide (3-EG, 3-glucuronide of estra-1,3,5-triene-3,17 ß-diol), dehydroepiandrosterone-3-glucuronide (DHEAG, 3-glucuronide of 5-androsten-3ß-ol-17-one), dehydroepiandrosterone (DHEA, 5-androsten-3ß-ol-17-one), and pregnenolone (PREG, 5-pregnen-3ß-ol-20-one), Steraloids Inc. (Newport, RI, USA) supplied pregnenolone glucuronide (PREGG, 3-glucuronide of 5-pregnen-3ß-ol-20-one), Fluka Chemie GmbH (Buchs, Italy) supplied corticosterone (CORT, 4-pregnen-11ß,21-diol-3,20-dione). Isopregnanolone glucuronide (IPG, 3-glucuronide of 5α-pregnan-3ß-ol-20-one), tetrahydrodeoxycosticosterone 21-glucuronide (21-THDG, 21-glucuronide of 5α-pregnan-3α,21-diol-20-one), corticosterone 21-glucuronide (21-CORTG, 21-glucuronide of 4-pregnen-11ß,21-diol-3,20-dione), cortisol 21-glucuronide (21-HCG, 21-glucuronide of 4-pregnen-11ß,17α,21-triol-3,20-dione), and d3-testosterone glucuronide were synthesised, purified, and characterized in our earlier studies [22-23]. Uridine-5'-diphosphoglucuronic acid (UDPGA, triammonium salt) and saccharic acid-1,4-lactone were purchased from Sigma-Aldrich, and NADPH (tetraammonium salt) from Roche (Indiapolis, USA). All reagents were at least reagent grade, and all solvents were HPLC grade.

Mouse brains. Animals Male outbred NMRI mice (specific pathogen free, bred locally at the Laboratory Animal Center, University of Helsinki), four to five weeks old for the seven-week nicotine treatment, were divided randomly into nicotine-receiving and control groups. The animals were housed in groups of four to ten animals at an ambient temperature of 20-22 ºC and a relative humidity of 50 ± 10% under a 12:12 light cycle (lights on at 0600 h). The mice had free access to mouse chow and drinking fluid. The mice received (-)-nicotine base (Fluka, Buchs, Switzerland) chronically. Nicotine was administered to the mice in their drinking water as the sole source of fluids for seven weeks. During the seven-week treatment, the nicotine concentration was increased stepwise from 50 to 300 µg/ mL during the first three weeks. Thereafter, the concentration remained constant at 300 µg/ mL until the end of the treatment. To make the solutions more palatable, hydrochloric acid was used to adjust

their pH to 6.8. The control mice drank tap water. The mice were decapitated in the afternoon of the 14th or 50th day of the oral nicotine treatment. The brains were rapidly removed from the skulls, rinsed with cold saline, and placed on an ice-cooled glass plate. The cortex, hippocampus, hypothalamus, and mid-brain were collected separately and frozen in cooled microcentrifuge tubes on dry ice, weighed, and then stored at -80 ºC. All procedures with animals described herein were performed according to the European Community Guidelines for the use of experimental animals and reviewed by the Animal Ethics Committee at the University of Helsinki.

Homogenization, extraction, and purification of the brain samples. The cortex, hippocampus, hypothalamus, and mid-brain samples were homogenized and purified; 20 µL of internal standard (5 nmol/ mL d3-testosterone glucuronide in ethanol) was added to the tissue samples. The brain tissue sample in 700 µL of ethanol/acetone mixture (1:1, v/v) was homogenized using ultra sonication for 0.5-1 min, amplitude 80%, interval 0.5-0.9 s (UP 50H, Dr. Hiescher, Germany). The samples were kept in an ice bath during homogenization. After homogenization, 300 µL of water was added to the mixture, which was then vortexed (15 s), ultrasonicated (10 min), and centrifuged (10 min, 16 400 g). The matrix was then re-extracted, and the combined supernatants were diluted with 10 ml of water prior to purification with a mixed-mode cation exchange cartridge (Oasis MCX 150 mg, 6 cc, Waters, MA, USA). The cartridges were conditioned with methanol and water (3 mL), and the samples were loaded and washed with 0.1 M HCl (1 mL) and water (3 mL). Steroids and respective conjugates were collected from the cartridge using acetonitrile/methanol (1:1 v/v, 4.5 mL). The supernatants were evaporated to dryness with nitrogen and re-constituted to 20 µL of methanol, and 180 µL of water was added.

Brain S9 Incubations. The animals whose brain tissue was used for S9 incubations were maintained in similar conditions to those described above for the nicotine-treated animals. The rats were males from the Wistar strain, and weighed 250-350 g. They were dissected after an experiment where the effect of a single intravenous dose of noradrenaline, adrenaline, isoprenaline, acetylcholine, and atropine on mean arterial pressure was tested under urethane anaesthesia. The mice were males from the C57BL/6 strain, aged three to six months, heterozygous with catechol-O-methyltransferase gene knock-out. They received no pharmacological manipulations.

The rats were still under urethane anaesthesia when sacrificed, whereas the mice were awake. To dissect the whole brain, the animals were decapitated. The brain was removed from the skull with forceps, frozen on dry ice, and stored at -80 °C until preparation of the S9 fractions.

The brain S9 fractions were isolated from whole mouse and rat brains with centrifugation [24]. The protein concentrations of the S9 fractions were determined with a commercial BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). The protein activity was 13.4 mg/ mL for the whole rat brain and 13.4 mg/ mL for the whole mouse brain. The incubations using the S9 fractions were carried out in a total volume of 250 µL containing 10 mM MgCl2, 5 mM saccharic acid-1,4-lactone, and 50 mM phosphate buffer, pH 7.4. The steroids (CORT, PREG, or DHEA) in dimethyl sulfoxide (DMSO) were added to a final concentration of 0.2 mmol/ L in the incubation mixtures. The concentration of DMSO in the reaction solution was 10% (v/v), and the protein concentration was 2.0 mg/ mL. The reactions were initiated by the addition of UDPGA to a final concentration of 5 mmol/ L, and reactions were carried out at 37 °C for 18 h. The reactions were terminated by the addition of 250 µL of methanol, and the

solutions were transferred to the ice bath. The mixtures were then centrifuged (15 min, 16 400 g), and the supernatants were collected. Residues were rinsed with 250 µL with 50% methanol (v/v), supernatants were combined, and internal standard (2.5 µL of 0.1 mmol/ L d3-TG in ethanol) was added to the solution. The brain S9 incubations were purified similarly to the brain extracts (Oasis MCX 60 mg, 6 cc, Waters, MA, USA). The supernatants were evaporated to dryness with N2, re-constituted to 250 µL of methanol/water (5:95, v:v), and subjected to CapLC-MS/MS analysis. Blank samples were prepared similarly to the brain S9 incubations: Blank 1 was incubated with cofactors and the S9 fraction (without steroids), Blank 2 was incubated with the S9 fraction alone, Blank 3 was incubated with cofactors and steroids (without the S9 fraction), and Blank 4 was incubated with cofactors alone.

Liquid chromatography-mass spectrometry. The HPLC was an Agilent 1100 Capillary LC containing an auto sampler with a 100-µL injection loop and Agilent 1100 quaternary pumps (Waldronn, Germany). The mass spectrometer used was an API 3000 triple-quadrupole instrument (Applied Biosystems/MDS Sciex, Toronto, ON, Canada) with a turbo ion spray source. Analyses were carried out using two columns: Symmetry Shield RP-18, 50 x 1 mm (column 1), and 100 x 0.3 mm (Waters, MA, USA) (column 2). The particle size in both columns was 3.5 mm, and the injection volume was 40 µl. A column switching technique was used to protect the analytical column and mass spectrometer from brain phospholipids. We used 50 mM NH4OAc (pH 4.5 with acetic acid) buffer/water (1:9, v/v) as a mobile phase A, and 50 mM NH4OAc (pH 4.5 with acetic acid) buffer/methanol/acetonitrile (1:3:6, v/v) as a mobile phase B. The sample transfer to column 1 and the washing of column 1 was performed within 7 min by using 5% eluent B at a flow rate of 20 µl/ min. After that, the sample was directed with column switching to the analytical column (column 2), and the separation was performed by a gradient elution as follows: 0 min 5% B, 0.1 min 45% B, 7 min 75% B, 10 min 85% B, and 11-29.5 min 100% B, and the equilibrium time between runs was 20 min. The flow rate was 9 µL/ min, and the temperature of the column was 50 ˚C. The ion spray voltage was 5500 V. Purified air (Atlas Copco, Belgium) was used as a nebulizing gas, and nitrogen (Generator 75-72, Whatman, Clifton, NJ) as curtain and collision gases. Neurosteroids and their metabolites were detected with a selected reaction monitoring (SRM) mode with dwell times of 150-250 ms, declustering potential 20-90 V, and collision energy 15-30 V. The monitored SRM pairs and structures of the compounds studied appear in Table 4.1. Relative retention times were calculated against d3-testosterone glucuronide (SRM 468 → 292). In the analysis of brain S9 incubations, the same method was used, except that the Symmetry Shield RP18 column (100 mm x 0.3 mm, 3.5 µm) was replaced with a Sun Fire RP18 column (100 mm x 0.3 mm, 3.5 µm) (Waters, MA, USA).

Table 4.1. *SMR pairs, declustering potential (DP, V), collision energy (CE, V), and structures of the steroid glucuronides studied.*

Compound **SRM pairs,** **m/z *(DP, CE)***	**Structure**	**Compound** **SRM pairs,** **m/z *(DP, CE)***	**Structure**
21-CORTG 523 → 329 (80, 30) 540 → 347 (25, 20)		IPG 512 → 301 (35, 17) 512 → 141 (35, 35)	
DHEAG 482 → 253 (30, 18) 482 → 271 (30, 35)		PREGG 510 → 299 (35, 20) 510 → 281 (35, 30)	
3-EG 466 → 273 (35, 20) 466 → 194 (35, 20)		21-THDG 528 →335 (20, 20) 528 →317 (20, 25)	
21-HCG 556 → 345 (20, 15) 539 → 345 (80, 15)		The structure of glucuronide (Glu) is:	

4.3 Results and discussion

The brain tissue samples from mice were dissected from the cortex, hippocampus, hypothalamus, and mid brain, and the steroid glucuronides were extracted from the tissue samples and analysed using capillary LC-ESI-MS/MS. The hippocampus, hypothalamus, and mid-brain samples were obtained from nicotine-treated and water-treated (control) mice, and the cortex sample only from water-treated mice. A seven-week nicotine treatment was administered in this study because previous research has shown that the administration of nicotine causes stress and increases the cerebrocortical and plasma concentrations of pregnenolone (PREG), progesterone (PROG), and allopregnanolone (AP) [25]. Because the concentrations of neurosteroids and their metabolites are very low in the mouse brain, we expected the concentrations of neurosteroid glucuronides to increase with nicotine treatment in the brain, thus facilitating the detection of glucuronides.

We developed a direct method for the qualitative analysis of intact neurosteroid glucuronides from biological samples. The detection and identification of intact steroid glucuronides from brain samples required the synthesis of reference compounds (glucuronide conjugates) and a highly sensitive and selective analytical method that could be achieved by combining an efficient sample preparation step and the capillary LC-ESI-MS/MS method. The authentic reference compounds were previously synthesised using rat liver microsomes [22]. To achieve sufficient selectivity for the analysis of low concentrations of neurosteroids and their metabolites, an efficient method for the removal of phospholipids is essential in order to minimize the ion suppression effect in ESI. We achieved this with solid-phase extraction (SPE) using a mixed-mode cation exchange cartridges. The optimized capillary LC separation conditions provided high resolution and minimal peak broadening (Figure 4.1). ESI in the positive ion mode with the selected reaction monitoring (SRM) in MS/MS measurements provided high sensitivity and selectivity. The analytes were eluted with relatively high content of organic solution that enhances the ionisation efficiency in ESI [26]. The glucuronides can also be ionized efficiently in a negative ion mode, but the MS/MS spectra of the deprotonated molecules showed mainly non-specific product ions derived from glucuronic acid moiety, whereas the MS/MS spectra of the protonated molecules or ammonium adduct ions in the positive ion mode showed more specific product ions formed by the loss of ammonia, water, and glucuronic acid moiety in various combinations. This is in agreement with the results obtained for glucuronides of anabolic steroids [27].

The SRM chromatograms of the brain extract samples show for the first time that neurosteroid glucuronide conjugates exist in the brain (Figure 4.1 and Table 4.2). Identification of the steroid glucuronides was based on the comparison of the relative retention times and relative abundances of two SRM pairs of each steroid glucuronide between the reference materials and the brain extract samples. The variation of the ratio of the relative abundances of the monitored product ions was less than 10% and the variation of the relative retention times was less than 2% in all positive identifications. Figure 4.1 shows that the SRM chromatograms of EG, 21-HCG, 21-CORTG and IPG fulfil the identification criteria. However, due to low concentration of 21-THDG the ratio of the relative abundances of the monitored product ions was higher than 10% compared to the reference sample and the identification of 21-THDG was not confirmed in that sample. Reagent blank samples, analysed at the beginning and end of the sample sequence, showed no memory effects (Figure 4.1). These results indicate that the identification of the neurosteroid glucuronides is reliable. It is worth of note, however,

that tetrahydroprogesterone (THPROG) has four isomers: IP (3β,5α), allopregnanolone (3α,5α), 3α,5β-THPROG, and 3β,5β-THPROG. Because the glucuronides of THPROG isomers are not commercially available and we were able to synthesise and characterize only IP glucuronide, it is possible that other glucuronides of THPROG isomers are not separated from IP glucuronide in the analysis of brain samples.

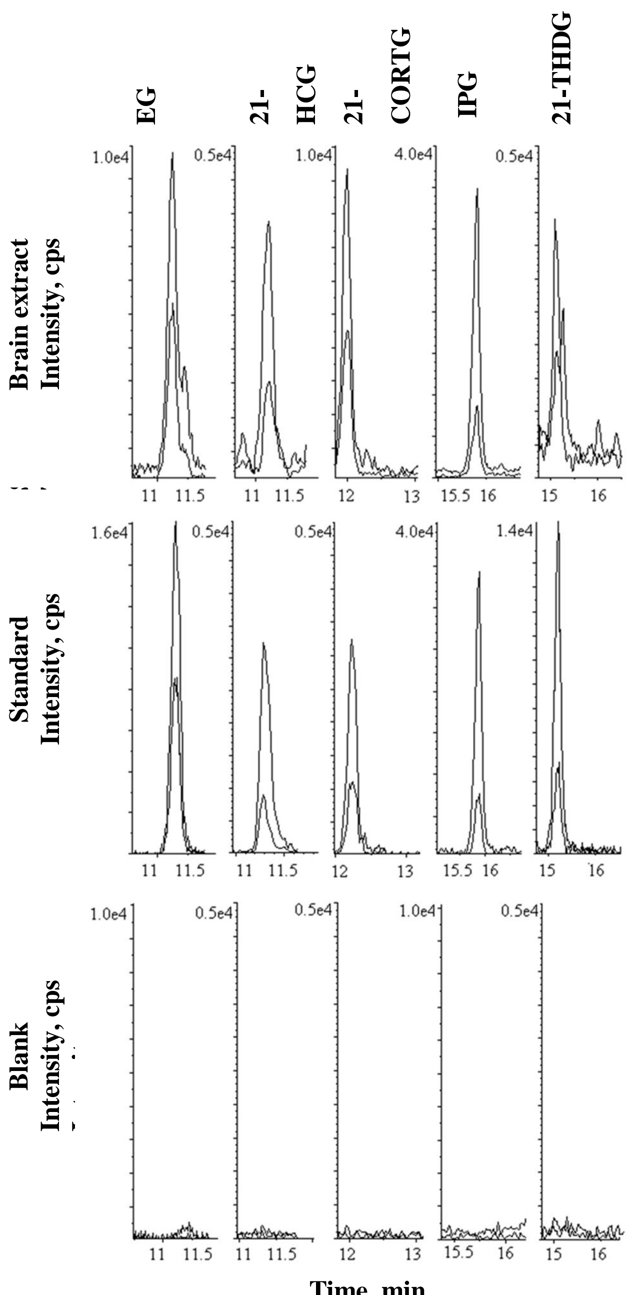

Figure 4.1. *Identification of neurosteroid glucuronide conjugates in the extracts from the hippocampus of mice treated with water for seven weeks (above), the reference material solution (middle) and the reagent blank (below). Each conjugate was detected using two SRM pairs (Table 4.1).*

Table 4.2. *A summary of steroid glucuronides identified from dissected areas of a mouse brain. The mice were treated for seven weeks with either nicotine or water.*

Brain area	Treatment	17-EG	21-HCG	21-CORTG	21-THDG	PREGG	IPG
Cortex	Water			X			X
Hippocampus	Nicotine	X	X	X	X	X	X
Hippocampus	Water	X	X	X		X	X
Hypothalamus	Nicotine	X	X	X			X
Hypothalamus	Water	X	X	X			
Mid-brain	Nicotine			X			
Mid-brain	Nicotine			X			
Mid-brain	Water			X			

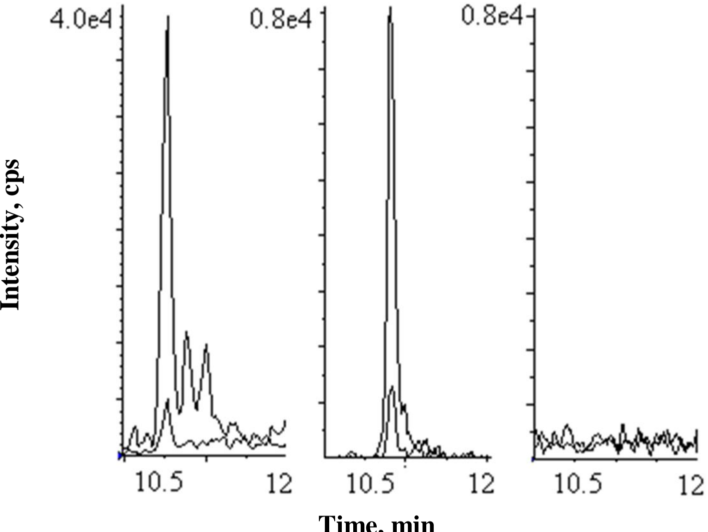

Figure 4.2. *SRM chromatograms of corticosterone glucuronide (21-CORTG) from an incubation sample of corticosterone with the S9 fraction of a whole mouse brain (left), a reference material solution (middle), and a blank sample (right, incubation of the S9 fraction of a whole mouse brain without corticosterone). The glucuronide was detected using two SRM pairs (Table 4.1).*

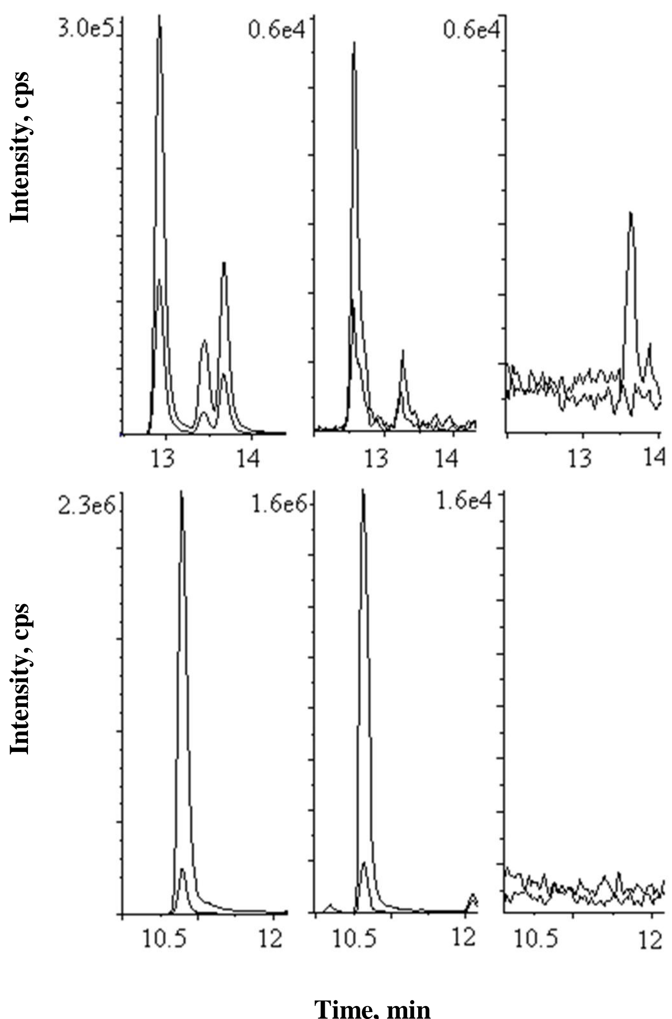

Figure 4.3. *SRM chromatograms of pregnenolone (PREGG) (above) and dehydroepiandrosterone glucuronides (DHEAG) (below) from incubation samples with the S9 fraction of a whole rat brain (left), a reference material solution (middle), and a blank sample (right, incubation of the S9 fraction of a whole rat brain with no aglycon). The glucuronides were detected using two SRM pairs (Table 4.1).*

The following neurosteroid glucuronides: 3-EG, 21-HCG, 21-CORTG, 21-THDG, PG, and IPG were detected from hippocampus samples, and 3-EG, 21-HCG, 21-CORTG, and IPG were detected in hypothalamus samples. IPG and 21-CORTG were detected in the extracts of the cortex, and 21-CORTG in the mid-brain extracts (Table 4.2). DHEAG was not detected in any of the samples. The only glucuronide detected in all the mouse brain samples was 21-CORTG, which is an interesting finding, since the secretion of corticosterone is a classical endocrine response to stress [28]. However, the peak areas of the glucuronides measured from the hippocampus of water- and nicotine-treated (stressed) mice showed no common behaviour. Note that the aim of this work was only to study qualitatively whether neurosteroid glucuronides exist in the brain. To study the effect of nicotine and stress on the concentrations of neurosteroids and their glucuronides in the brain requires the development and validation of a quantitative method, which is the aim of the following study.

To study whether neurosteroid glucuronides are biosynthesised in the rodent brain, CORT (at a concentration of 0.2 mmol/ L) was incubated in vitro with an S9 fraction of a whole mouse and rat brain, and PREG and DHEA were similarly incubated with an S9 fraction of a whole rat brain. The concentration of cofactor UDPGA was 5 mM in the incubations. The S9 fraction is a subcellular fraction containing both cytosol and microsomes, and is well suited for in vitro metabolism studies of both phase I and phase II biotransformations. The SRM chromatograms of the incubation samples as well as the blank samples (Figures 4.2-4.3) demonstrate unambiguously that glucuronides 21- CORTG, PREGG, and DHEAG formed in the incubations, indicating that the formation of neurosteroid glucuronides is possible in the rodent brain. The SRM chromatograms of CORTG (Figure 4.2) and PREGG (Figure 4.3, above) of the incubation samples show extra peaks which can be isomers of COTRG and PREGG. However, their identity was not possible to confirm due to lack of reference compounds.

4.4 Conclusions

A direct method for the qualitative analysis of neurosteroid glucuronides in the mouse brain was developed using a capillary LC-ESI-MS/MS technique. Six neurosteroid glucuronide conjugates were identified from eight brain extract samples of mouse cortex, hippocampus, hypothalamus, and mid-brain. This study also showed with in vitro incubations using S9 fractions of a whole mouse and rat brain that the formation of neurosteroid glucuronides is possible at least in the rodent brain. Our results raise the urgent need for quantitative methods for the analysis of neurosteroids and their glucuronide conjugates in the brain. Although the role of steroid glucuronidation in the brain remains unknown, several questions emerge: Is glucuronidation an elimination pathway for steroids in the brain? Do efflux transporters exist for steroid glucuronides? Are glucuronides back-hydrolysed to native steroids or do they cumulated to the brain? Have they neurotoxic or neuroprotective properties? Do neurosteroid glucuronides yield some pharmacological activity in the brain? Answering to such questions requires thorough pharmacological experiments and analytical measurements.

4.5 Acknowledgement

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4.6 References

[1] B. Stoffel-Wagner, Neurosteroid Biosynthesis in the Human Brain and Its Clinical Implications, Ann. NY Acad. Sci. 1007 (2003) 64-78.
[2] D. Belelli, J.L. Lambert, Neurosteroid: endogenous regulators of the GABAA receptor, Nat. Rev. Neurosci. 6 (2005) 565-575.
[3] M. Vallee, W. Mayo, G. Koob, M. Le Moal, Neurosteroids in learning and memory processes, International Review of Neurobiology 46 (2001) 273-320.
[4] S.H. Mellon, L.D. Griffin, Neurosteroids: biochemistry and clinical significance. Trends Endocrinol. Met.. 13 (2002) 35-43.
[5] B. Stoffel-Wagner, Neurosteroid metabolism in the human brain, Eur. J. Endocrinol. 45 (2001) 669-79.
[6] A. Mensah-Nyagan, J.L. Do-Rego, D. Beaujean, V. Luu-The, G. Pelletier, H. Vaudry, Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosyntheis in the central nervous system, Pharmacol. Rev. 51 (1999) 63-81.
[7] M. Shimizu, H. Tamura, Identification and localization of two hydroxysteroid sulfotransferases in the brain, J. Health Sci. 48 (2002) 467-472.
[8] M. Shimada, K. Yoshinari, E. Tanabe, E. Shimakawa, M. Kobashi, K. Nagata, Y. Yamazoe, Idenfication of ST2A1 as a rat brain neurosteroid sulfotransferase mRNA, Brain Res. 920 (2001) 222-225.
[9] M. Shimizu, Y. Nakano, T. Yamasaki, T. Kimoto, S. Kawato, H. Tamura, Regio- and isoform-specific expression of hydroxysteroid sulfotransferases in rat brain, J. Health Sci. 50 (2004) 689-92.
[10] C.D. King, G.R. Rios, J.A. Assouline, T.R. Tephly, Expression of UDP-glucuronosyltransferases (UGTs) 2B7 and 1A6 in the human brain and identification of 5-hydroxytryptamine as a substrate Arch. Biochem. Biophys. 365 (1999) 156-162.
[11] F.G. Suleman, A. Abid, D. Gradinaru, J.L. Daval, J. Magdalou, A. Minn, Identification of the uridine diphosphate glucuronosyltransferase isoform UGT1A6 in rat brain and in primary cultures of neurons and astrocytes, Arch. Biochem. Biophys. 358 (1998) 63-67.
[12] G. Belanger, O. Barbier, D. Hum, A. Belanger, Molecular cloning, expression and characterization of a monkey steroid UDP-glucurosyltransferases, UGT2B19, that conjugates testosterone, Eur. J. Biochem. 260 (1999) 701-708.
[13] Y. Akwa, E.E. Baulieu, Dehydroepiandrosterone sulfate and dehydroepiandrosterone: neuroactive steroids, Curr. Opin. Endocrinol. Diabetes 7 (2000) 160-167.
[14] M. Schumacher, P. Liere, Y. Akwa, K. Rajkowski, W. Griffits , K. Bodin , J. Sjövall, E.E. Baulieu EE, Pregnenolone sulfate in the brain: A controversial neurosteroid, Neurochem. Int. 52 (2008) 522-540.
[15] M. Schumacher, S. Weill-Engerer, P. Liere, F. Robert, R.J.M. Franklin, L.M. Garcia-Segura, J.J. Lambert, W. Mayoe, A. Parducz, U. Suter, C. Carelli, E.E. Baulieu, Y. Akwa, Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog. Neurobiol. 71 (2003) 3-29.

[16] C. Corpechot, P. Robel, M. Axelson, J. Sjövall, E.E. Baulieu, Characterization and measurement of dehydroepiandrosterone sulfate in rat brain, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 4704-4707.
[17] C. Corpechot, M. Synguelakis, S. Talha, M. Axelson, J. Sjövall, R. Vihko, E. Baulie, P. Robel, Pregnanolone and its sulphate ester in rat brain. Brain Res. 270 (1983) 119-125.
[18] P. Liere, A. Pianos, B. Eychenne, A. Cambourg, S. Liu, W. Griffiths, M. Schumacher, J. Sjövall, E.E. Baulieu, Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain, J. Lipid Res. 45 (2004) 2287-2302.
[19] S. Liu, J. Sjövall, W.J. Griffiths, Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography−electrospray mass spectrometry, Anal. Chem. 75 (2003) 5835-5846.
[20] P. Uutela, L. Karhu, P. Piepponen, M. Käenmäki, R.A. Ketola, R. Kostiainen, Discovery of dopamine glucuronide in rat and mouse brain microdialysis samples using liquid chromatography tandem mass spectrometry, Anal. Chem. 81 (2009) 427-434.
[21] P. Wang, O. Kuchel, N. Buu, J. Genest, Catecholamine glucuronidation: an important pathway for dopamine in the rat, J. Neurochem. 40 (1983) 1435-1440.
[22] S. Jäntti, A. Kiriazis, R. Reinilä, R. Kostiainen, R.A. Ketola, Enzyme-assisted synthesis and charaterization of neuroactive steroid glucuronides, Steroids 72 (2007) 287-296.
[23] T. Kuuranne, O. Aitio, M. Vahermo, E. Elovaara, R. Kostiainen, Enzyme-assisted synthesis of glucuronide conjugates of methyl-testosterone (17α-methylandrost-4-en-17β-ol-3-one) and nandrolone (estr-4-en-17β-ol-3-one) metabolites, Bioconjugate Chem. 13 (2002) 194-199.
[24] L. Luukkanen, E. Elovaara, P. Lautala, J. Taskinen, H. Vainio, Characterization of 1-hydroxypyrene as a novel marker substrate of 3-methylcholanthrene-inducible phenol UDP-glucuronosyltransferase(s), Pharmacol. Toxicol. 80 (1996) 152-158.
[25] A. Concas, C. Sogliano, P. Porcu, C. Marra, A. Brundu, G. Biggio, Neurosteroids in nicotine and morphine dependence, Psychopharmacology 186 (2006) 281-292.
[26] R. Kostiainen and A.P. Bruins, Effect of solvent on dynamic range and sensitivity in pneumatically assisted electrospray (ion spray) mass spectrometry, Rapid Commun. Mass Spectrom. 10 (1996) 1393-1399.
[27] T. Kuuranne, M. Vahermo, A. Leinonen, R. Kostiainen, Electrospray and atmospheric pressure chemical ionization tandem mass spectrometric behavior of eight anabolic steroid glucuronides. J. Am. Soc. Mass Spectrom. 11 (2000) 722-730.
[28] L. Sapolsky, L.M. Romero, A.U. Munck, How do glucocorticoids influence astress responses? Integrating permissive, suppressive, stimulatory, and preparative actiuons. Endocr. Rev. 21 (2000) 55-89.

5 Determination of Steroids and Their Intact Glucuronide Conjugates in Mouse Brain by Capillary Liquid Chromatography-Tandem Mass Spectrometry

A method for the identification and quantitation of 10 neurosteroids, and their 2 sulfate and 9 glucuronide conjugates in mouse brain tissues was developed and validated. The method includes the extraction of homogenized brain by SPE, and the analysis of the extracts by capillary liquid chromatography-tandem mass spectrometry. The main advantage of the method is that steroid conjugates in brain can be analyzed as intact compounds, without derivatization, hydrolysis, or complex sample preparation procedures; thus the true identity of the conjugates can be confirmed with tandem mass spectrometric detection. The method was validated to show its linearity ($r > 0.998$) and precision (RSD < 9%). The limits of detection in solution were from 6 to 80 pmol/L for steroid glucuronides, from 13 to 32 pmol/L for steroid sulfates, and from 26 pmol/L to 2.2 nmol/L for native steroids. The recovery of internal standards was 95 % for d3-testosterone glucuronide and 69 % for d4-allopregnanolone from spiked mouse hippocampus. Brain tissues samples from mouse hippocampus and hypothalamus were analyzed using the new method. Seral steroids and glucuronides were identified and quantified from the mouse brain at concentration levels of 0.2-58 ng/g. The concentrations of steroid glucuronides were significantly higher compared to those of their aglycons, indicating that glucuronidation can be an important metabolic pathway for some steroids in the mouse brain. The method developed in this study provides for the first time direct quantitative determination of steroids and their glucuronides and sulfates in brain without hydrolysis, and therefore creates the possibility to study in detail the role of steroid glucuronidation and sulphation in the brain.

5.1 Introduction

Steroids in brain are divided into subclasses: neurosteroids, non-active and neuroactive peripheral steroids. For clarity reasons a term 'neurosteroid' is used for all subclasses in this paper. At a biochemical level, neuroactive steroids have been shown to be involved in the modulation of several reseptors, such as $GABA_A$, N-methyl-d-aspartate (NMDA), and sigma receptors.[1-4] In phase I metabolism, steroids are biochemically converted to other steroids by the oxidation, hydroxylation or reduction of functional groups, which usually increases the polarity of the compound and serves sites for phase II conjugation. Phase I metabolism and the biosynthesis of steroids are well documented and reviewed.[1,4-7] Phase II metabolism includes the formation of glucuronide and sulfate conjugates, which promotes their excretion. The presence of sulfated steroids in the brain of mammals is still a matter of controversy although pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS) have been detected in the rat brain using indirect methods.[8-9] Because the steroid sulfates were hydrolyzed before analysis, some researchers suspect that these sulfate conjugates are derived at least in part from other origins.[10-12] Glucuronidation is the most common conjugation and inactivation reaction of steroids in the liver and uridine diphosphate glucuronosyl transferases (UGTs) 1A6,[*13-14*] 2A1,[15] 2B7,[13] and 2B19 [16] have been found in the brain also. No previous studies have explored the role of the glucuronidation of steroids in the brain, most obviously due to the lack of sufficiently sensitive and selective methods for the direct analysis of neurosteroid glucuronides.

However, steroid glucuronides have been recently discovered the mouse brain.[17] Also glucuronides of neurotransmitters, such as dopamine, serotonin, and their metabolites, have been detected in brain microdialyte samples with LC-MS/MS at low nM levels.[18,19] Detection limits in the nmol/L range have been reported previously for LC-MS analysis of steroid glucuronide conjugates other than neurosteroids glucuronides.[20-22]

Radioimmunological assays (RIA) and gas chromatography-mass spectrometry (GC-MS) have traditionally been used for the detection and quantification of neurosteroids. RIA is relatively sensitive (7 – 15 pg), but the lack of selectivity and cross reactions are major disadvantages.[23] GC-MS provides both a sensitive and selective method for steroids in brain extracts, achieving detection limits (DLs) as low as 1 – 5 pg (0.3 – 1.5 ng/g) in the brain.[24,25] However, GC-MS as well as RIA precludes the direct analysis of steroid conjugates, and a complicated sample pre-treatment procedure is needed, including hydrolysis, derivatisation and extensive purification schemes such as liquid chromatographic (LC) fractionation or several solid-phase extraction (SPE) steps.[23,24,26]

Liquid chromatography combined with atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI) mass spectrometry has been shown to be a very useful method for the analysis of neurosteroids and their metabolites. However, to our knowledge current LC-MS methods are based on the derivatisation of neurosteroids, which improve sensitivity 20- to 150-fold compared to the analysis of steroids without derivatisation using APCI-MS. The most common derivatisation agents used in the derivatisation of steroids are Girard P reagent (GP),[27,28] 2-nitro-4-trifuoromethylphenylhydrazine (NFPH),[29] and 2-hydrazino-1-methylpyridine (HMP).[30,31] DLs below 1 ng/g have been demonstrated for HMP derivatives in rat brain extracts using ESI in the positive ion mode for example, or for NFPH derivatives using electron capture atmospheric pressure chemical ionization (ECAPCI).[29] Using LC-ESI-MS and HMP derivatisation, Higashi *et al.* determined allopregnanolone (AP), epiallopregnanolone, and 5α-dihydroprogesterone (DHP) levels in the brains of stressed rats to be 0.2 – 5 ng/g in tissue, but the levels in untreated rats were less than the limits of quantitation.[30] Higashi's group also showed, using LC-ECAPCI-MS, that pregnenolone (PREG) and progesterone (PROG) levels were significantly higher in stressed rat brains (5 – 70 ng/g and 0 – 22 ng/g, respectively) than in the brains of control rats (P: 5 – 10 ng/g, PROG: < DL). Griffiths *et al.* detected 0.04 – 38 ng/g of ketonic neurosteroids as their oxime derivatives in rat brain extracts using nanoLC-ESI-MS-MS.10 They also applied the method for a direct analysis of PREG and DHEA sulfates; no sulfates were found, however (DLs were 0.3 ng/g). These results were consistent with the earlier results presented by Higashi *et al.*[32] but contradicted with the results presented Corpechot *et al.*[8,9] Griffiths' and Higashi's methods measured intact sulfate conjugates directly whereas Corpechot *et al.* measured sulfates after hydrolysis.[8,9] Consequently, steroids detected after hydrolysis may be derived from conjugates other than those derived from sulfates. This highlights the need for direct methods in the analysis of neurosteroid conjugates.

We recently reported the discovery of steroid glucuronides in the mouse brain,[17] but thus far no quantitative method has been presented that provides direct analysis of neurosteroids and their intact sulfate and glucuronide conjugates in brain samples without derivatisation, most obviously because the required sensitivity level is very difficult to achieve. The derivatisation step excludes the development of a universal neurosteroid analysis method, because derivatisation can occur only to those steroids having a specific functional group, such as carbonyl. Consequently current methods are able to analyze only a few steroids at a time, so a more universal method that could measure different classes of steroids within one run is urgently needed. In this study, we present the development and validation of a capillary LC-ESI-MS-MS method for the analysis of 10 steroids, and their 2 sulfate and 9 glucuronide conjugates in brain tissues without derivatisation and hydrolysis. The method was applied to the analysis of four authentic samples from mouse brain.

5.2 Experimental

Reagents and standards. Cortisol (HC, 4-pregnen-11ß,17α,21-triol-3,20-dione), dehydroepiandrosterone (DHEA, 5-androsten-3ß-ol-17-one), 5α-dihydroprogesterone (DHP, 5α-pregnan-3,20-dione), estradiol (E, 1,3,5-estratriene, 3,17 ß-diol), isopregnanolone (IP, 5α-pregnan-3ß-ol-20-one), pregnenolone (PREG, 5-pregnen-3ß-ol-20-one), progesterone (PROG, 4-pregnane-3,20-one), testosterone (T, 4-androsten-17ß-ol-3-one), 5α-tetrahydrodeoxycorticosterone (THD, 5α-pregnan-3α,21-diol-20-one), dehydroepiandrosterone 3-glucuronide (DHEAG), estradiol 17-glucuronide (17-EG), dehydroepiandrosterone 3-sulfate (DHEAS) and pregnenolone 3-sulfate (PREGS) standards were supplied from Sigma (St. Louis, MO, USA); corticosterone (CORT, 4-pregnen-11ß,21-diol-3,20-dione) was supplied from Fluka Chemie GmbH (Buchs, Italy); testosterone glucuronide (TG) and pregnenolone glucuronide (PREGG) from Steraloids Inc. (Newport, RI, USA) and d4-allopregnanolone (d4-AP, d4-5α-pregnan-3α-ol-20-one) was supplied from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Isopregnanolone glucuronide (IPG), tetrahydrodeoxycorticosterone 3- and 21-glucuronides (THDGs), corticosterone 21-glucuronide (21-CORTG), cortisol 21-glucuronide (21-HCG) and d3-testosterone glucuronide (d3-TG) were synthesised, purified and characterized according to our previous studies.[33-35] The structures and biosynthesis routes of steroids and their conjugates studied in this work appear in Figure 5.1. All reagents were reagent grade and all the solvents were HPLC grade.

Animals. Male outbred NMRI mice (specific pathogen free, bred locally in the Laboratory Animal Center, University of Helsinki), four to five weeks old for the seven-week nicotine treatment and eight to ten weeks old for the two-week nicotine treatment, which were used for the analysis and method validation, respectively. Animals were housed in groups of four to ten animals at an ambient temperature of 20 – 22 ºC and a relative humidity of 50 ± 10 % under a 12:12 light cycle (lights on at 0600 h). The mice had free access to mouse chow and drinking fluid. All procedures with animals were performed according to European Community Guidelines for the use of experimental animals and reviewed by the Animal Ethics Committee at the University of Helsinki.

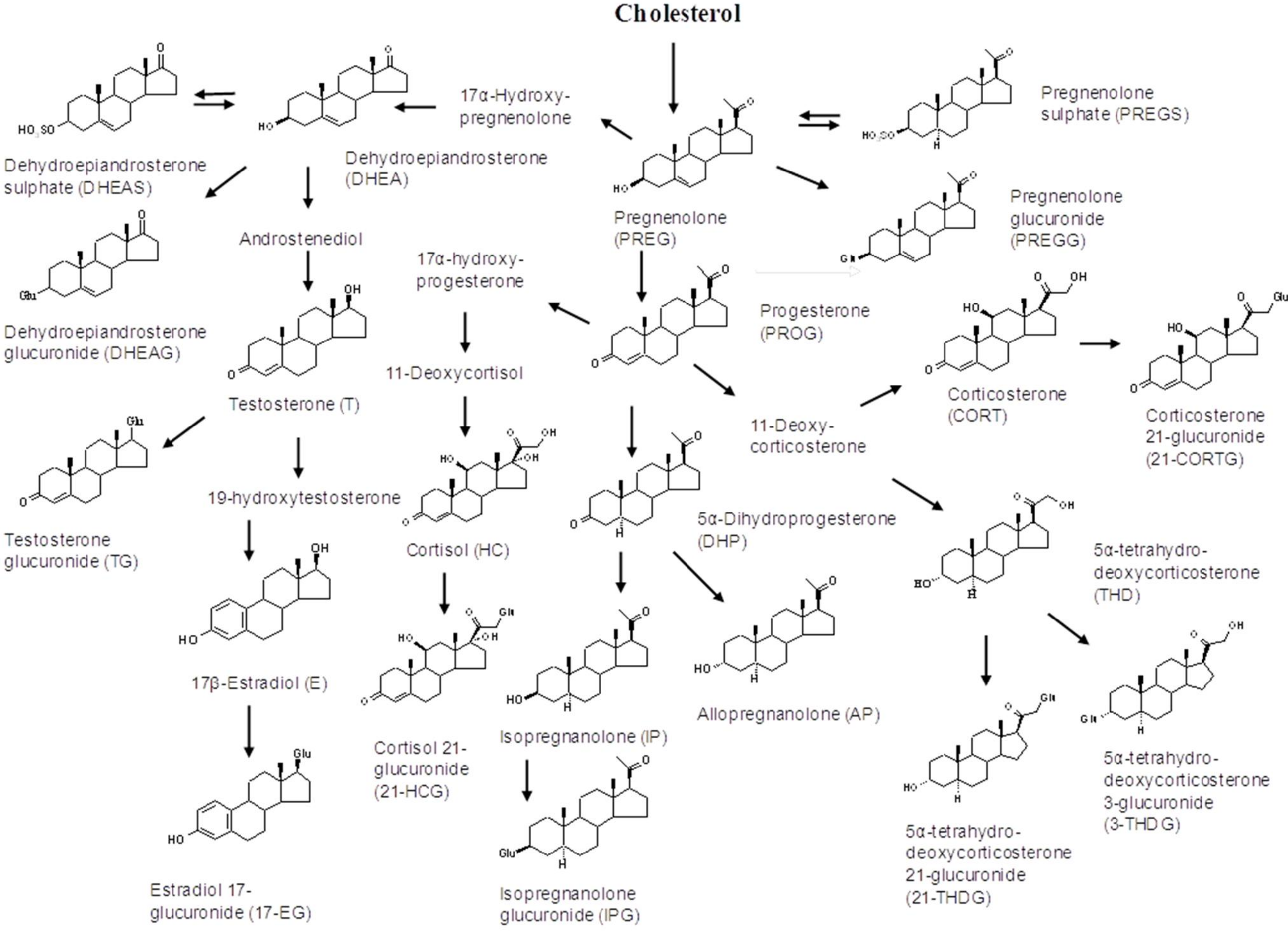

Figure 5.1. *Structures and abbreviations of studied steroids, and their sulfate and glucuronide conjugates, showing their respective biosynthesis routes. The structure of glucuronide is*

Drug treatments. The drug treatment has been previously described.[18] Shortly, the mice received (-) -nicotine base (Fluka, Buchs, Switzerland) chronically. Nicotine was administered to mice through their drinking water as the sole source of fluid for two or seven weeks. In the two-week treatment, the concentration of nicotine solution was 200 mg/mL throughout the entire treatment. In the seven-week treatment, the nicotine concentration was increased stepwise from 50 to 300 mg/mL during the first three weeks. Thereafter, the concentration remained constant at 300 mg/mL until the end of the treatment. pH of the solutions was adjusted to 6.8 with hydrochloric acid. The control mice drank tap water. The mice were decapitated in the afternoon of day 14 or day 50 of the oral nicotine treatment. The brains were rapidly removed from the skulls, rinsed with cold saline and placed on an ice-cooled glass plate. The hippocampus (2- and 7-week treatments) and hypothalamus (7-week treatment) were collected and frozen in cooled microcentrifuge tubes on dry ice, weighed, and stored at -80 ºC.

Homogenization, extraction and purification of the brain samples. Internal standards (20 µL of a mixture of 5 nmol/L d3-testosterone glucuronide and 500 nmol/L d4-allopregnanolone in ethanol, which corresponds to a concentration of 1 nmol/L and 100 nmol/L per sample, respectively) were added to each brain sample one hour before homogenization to mimic the natural absorption of compounds into the brain matrix. Homogenization was performed in 700 µL of an ethanol: acetone mixture (1:1, v/v) using ultrasonication for 0.5 – 1 min, amplitude 80%, and interval 0.5 – 0.9 s (UP 50H, Dr.Hiescher, Germany) in an ice bath. After homogenization, 300 µL of water was added to the mixture, which was then vortexed (15 s), ultrasonicated (10 min) and centrifuged (10 min, 16 400 g). The matrix was re-extracted with 300 µL of an ethanol: acetone (1:1, v/v): water mixture (7:3 v/v), and the combined supernatants were diluted with 10 mL of water and purified with a mixed mode cation exchange cartridge (Oasis MCX 150 mg, 6 cc, Waters, MA, USA). The cartridges were conditioned with methanol and water (3 mL), and the samples were loaded and washed with 0.1 M HCl (1 mL) and water (3 mL). Steroids and respective conjugates were collected from the cartridge with a mixture of acetonitrile/methanol (1:1 v/v, 4.5 mL). After centrifugation, the supernatants were evaporated to dryness with N_2, reconstituted to 10 µL of methanol and 90 µL of water was added.

Liquid chromatography-mass spectrometry. The HPLC system consisted of an Agilent 1100 binary pump, an Agilent 1100 Capillary LC equipped with an auto sampler using a 100-µL injection loop (Agilent, Waldronn, Germany). The mass spectrometer was an API 3000 triple quadrupole instrument (Applied Biosystems/MDS Analytical Technologies, Toronto, ON, Canada) with a turbo ion spray source. Analyses were carried out using two endcapped Symmetry Shield RP-18 columns: 50 x 1 mm ID, 3.5 µm (column 1), and 100 x 0.3 mm ID, 3.5 µm (column 2) (Waters, MA, USA). The injection volume was 40 µL. The column switching was used to protect column 2 and mass spectrometer from brain phospholipids. The separation was performed with a gradient using 50 mM ammonium acetate (NH_4Ac) (pH 4.5 with acetic acid) buffer/water (1:9, v/v) as mobile phase A and 50 mM NH_4Ac (pH 4.5 with acetic acid) buffer/methanol/acetonitrile (1:3:6, v/v) as mobile phase B. The sample transfer to column 1 and the washing of the column was performed within 7 min using 5% eluent B and a flow rate of 20 µL/min. The sample was then directed by column switching to the column 2. The gradient program was 0 min 5% B, 0.1 min 45% B, 7 min 75% B, 10 min 85% B and 11 – 29.5 min 100% B, and the equilibrium time between runs was 20 min. The flow rate was 9 µL/min, and the temperature of the column 50 ˚C.

Table 5.1. *Relative retention times of compounds and MS/MS operating parameters. d3-testosterone glucuronide served as an internal standard for all conjugates, cortisol and corticosterone (analytes in periods 1-5), and d4-allopregnanolone for the others (analytes in periods 6-8). Abbreviations for compounds appear in Figure 5.1*

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Period	Compound	Relative retention time (min)	SRM pair	Precursor ion	Product ion	DP[1] (V)	CE[2] (V)
1	17-EG	0.94	466→ 273	$[M+NH_4]^+$	$[M+H-Glu]^+$	35	20
			466→ 194	$[M+NH_4]^+$	unknown	35	20
1	21-HCG	0.94	556→ 345	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	15
			539→ 345	$[M+H]^+$	$[M+H-Glu-H_2O]^+$	80	15
2	TG	1.00	482→ 465	$[M+NH_4]^+$	$[M+H]^+$	25	15
			465→ 289	$[M+H]^+$	$[M+H-Glu]^+$	90	27
2	d3-TG	1.00	468→ 292	$[M+H]^+$	$[M+H-Glu]^+$	90	27
2	21-CORTG	1.00	523→ 329	$[M+H]^+$	$[M+H-Glu-2H_2O]^+$	80	30
			540→ 347	$[M+NH_4]^+$	$[M+H-Glu]^+$	25	20
2	DHEAG	1.05	482→ 271	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	30	18
			482→ 253	$[M+NH_4]^+$	$[M+H-Glu-2H_2O]^+$	30	35
3	HC	1.13	363→121	$[M+H]^+$	From the A-ring	25	20
			363→327	$[M+H]^+$	$[M+H-2H_2O]^+$	25	22
3	3-THDG	1.12	528→335	$[M+NH_4]^+$	$[M+H-Glu]^+$	20	20
			528→317	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	25
4	21-THDG	1.21	528→335	$[M+NH_4]^+$	$[M+H-Glu]^+$	20	20
			528→317	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	25
4	PREGG	1.22	510→ 299	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	35	20
			510→ 281	$[M+NH_4]^+$	$[M+H-Glu-2H_2O]^+$	35	30
4	CORT	1.26	364→ 347	$[M+NH_4]^+$	$[M+H]^+$	25	25
			347→ 121	$[M+H]^+$	from the A-ring	25	30
4	IPG	1.28	512→ 301	$[M+NH_4]^+$	$[M+H-H_2O]^+$	35	17
			512→ 141	$[M+NH_4]^+$	$[Glu+H-2H_2O]^+$	35	35
4b	DHEAS	1.22	367→ 97	$[M-H]^-$	$[H_2SO_4-H]^-$	-60	-60
			367→80	$[M-H]^-$	$[^-SO_3]^-$	-60	-125
5	PREGS	1.44	395→ 97	$[M-H]^-$	$[H_2SO_4-H]^-$	-60	-60
			395→80	$[M-H]^-$	$[^-SO_3]^-$	-60	-125
6	T	0.80	289→ 109	$[M+H]^+$	from the A-ring	30	40
			289→ 97	$[M+H]^+$	from the A-ring	30	40
6	DHEA	0.84	306→ 271	$[M+NH_4]^+$	$[M+H-H_2O]^+$	27	15
			306→ 253	$[M+NH_4]^+$	$[M+H-2H_2O]^+$	27	18
6	E	0.84	290→ 273	$[M+NH_4]^+$	$[M+H]^+$	25	9
			290→ 273	$[M+NH_4]^+$	unknown	25	32
7	THD	0.88	352→ 335	$[M+NH_4]^+$	$[M+H]^+$	30	12
			352→ 317	$[M+NH_4]^+$	$[M+H-H_2O]^+$	30	17
7	PROG	0.99	315→ 97	$[M+H]^+$	from the A-ring	50	35
			315→ 109	$[M+H]^+$	from the A-ring	50	35
8	DHP	0.95	334→ 317	$[M+NH_4]^+$	$[M+H]^+$	25	15
			317→ 299	$[M+H]^+$	$[M+H-H_2O]^+$	80	15
8	PREG	0.98	334→ 317	$[M+NH_4]^+$	$[M+H]^+$	25	15
			317→ 299	$[M+H]^+$	$[M+H-H_2O]^+$	80	15
8	IP	0.98	336→ 301	$[M+NH_4]^+$	$[M+H-H_2O]^+$	60	30
			336→ 283	$[M+NH_4]^+$	$[M+H-2H_2O]^+$	60	15
8	d4-AP	1.00	340→305	$[M+NH_4]^+$	$[M+H-H_2O]^+$	60	30

[1] DP Declustering potential, [2] CE Collision energy.

The ion spray voltage was 5500 V in the positive ion mode and -4200 V in the negative ion mode. Purified air (Atlas Copco, Belgium) served as a nebulizing gas, and nitrogen (Generator 75-72, Whatman, Clifton, NJ) as curtain and collision gases. Steroids and their metabolites were detected by selected reaction monitoring (SRM) with dwell times of 150-250 ms. The precursor ions and the monitored product ions appear in Table 5.1. For brain extracts, d3-testosterone glucuronide served as an internal standard for the steroid conjugates, cortisol and corticosterone, and d4-allopregnanolone for other non-conjugated steroids. Quantitation was performed using an internal calibration method. The concentrations of d4-allopregnanolone and d3-testosterone glucuronide in the final samples were 100 nmol/L and 1 nmol/L, respectively.

5.3 Results and discussion

The aim of this study was to develop a quantitative method for determination of steroids and their sulphate and glucuronide conjugates from brain tissues in one LC-MS/MS run. Challenges in the analysis of steroids in brain tissue samples are mainly high sensitivity and selectivity requirements combined with a difficult matrix containing high amounts of endogenous compounds, such as various lipids. The sample preparation method presented by Liu *et al.*[10] was selected for the starting point in our method development, in which they extracted neurosteroids from rat brain samples using ethanol, and the extracts were purified with several steps of solid-phase extraction (SPE). In our experiments changing the extraction solvent from ethanol to ethanol/acetone (1:1 v/v) achieved sufficient extraction efficiency, and the amount of interfering phospholipids reduced in the extracts. The sample was further purified with SPE using a mixed-mode cation exchange cartridge. A column switching technique was used for further sample purification. Liere *et al.* have shown that unreliable steroid isolation and solvolysis procedures resulted in errors in case of steroid sulfates or steroids with a 3β-hydroxy-Δ^5 structure, when harsh conditions as high temperature or strong reagents, such as sodium hydroxide or triethylamine/heptafluorobutyric anhydride (HFBA), were used.[12] We used only mild conditions and solvents (room temperature and common organic solvents), and derivatization or solvolysis were not used. Therefore, we assume that the risk for cholesterol autoxidation was minimal. Mild conditions are also needed because the O-glycosidic bond is a weak bond, and glucuronide conjugates are easily decomposed if harsh conditions are used. Good recovery of the internal standard (d3-TG) through the sample preparation process showed preservation of the glucuronide conjugates. Furthermore, also sulfate conjugates preserved during the sample preparation step, as the signal intensities of sulfates spiked to brain homogenates were at the same level as those in spiking solutions (data not shown).

The feasibility of methanol, acetonitrile, and a combination of them was tested as an organic solvent in the LC-ESI-MS/MS analysis. Acetonitrile provided better resolution in LC separation, but lower ionisation efficiency in ESI, than methanol. The acetonitrile/methanol (2:1 v/v) mixture as a compromise provided practically the same sensitivity as methanol and preserved nearly the same resolution in LC separation as acetonitrile. Optimal resolution was achieved at a column temperature of 40 – 50 ºC. With on-column concentration it was possible to increase sensitivity by injecting 40 µL of sample solutions without band broadening. The peaks were narrow, with peak widths at half-height varying between 0.1 and 0.15 min, and minimal peak tailing was observed (Fig. 2).The repeatability of the retention times was good, as the relative standard deviation (RSD) was less than 1 % for internal standards (mouse hippocampus, n = 10).

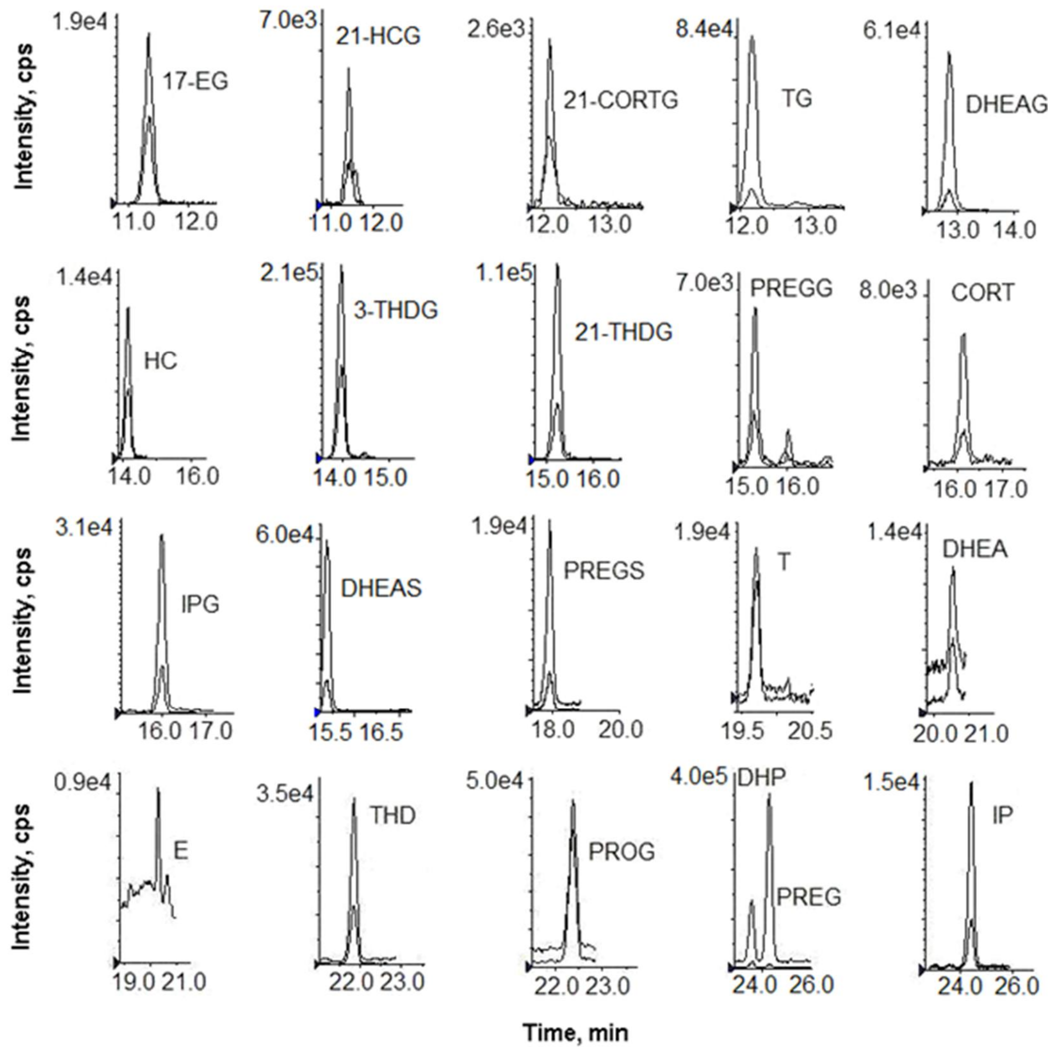

Figure 5.2. *An SRM chromatogram of a standard run. Abbreviations for compounds appear in Figure 5.1, and SRM pairs and relative retention times in Table 5.1.*

Steroids and their glucuronides were detected in the positive ion mode, and steroid sulfates in the negative ion mode. The mass spectra of the steroids and their glucuronides showed abundant protonated molecules or ammonium adduct ions or both with minimal fragmentation. The protonated molecule dominated over the ammonium adduct only if the structure of the analyte had a conjugated carbonyl group in the A-ring, as also recognized in the previous work with anabolic steroids.[36] The ammonium adducts were relatively unstable and can be dissociated at elevated ESI temperatures. To avoid the dissociation of ammonium adducts and to obtain good sensitivity, the ESI was operated at room temperature. Also other parameters, such as pH and concentration of the buffer solution, affecting the formation of ammonium adducts, were carefully optimized. The steroid sulfates produced abundant deprotonated molecules without fragmentation. In MS/MS analysis, the product ions monitored for non-conjugated steroids were formed either by the loss of ammonia or water or both, using low collision energy (15 V), or by the dissociation

of the A-ring, producing mainly product ions at *m/z* 97, 109, or 121, using high collision energy (40 V). The use of low collision energy provides high sensitivity, since no extensive fragmentation occurs and the ion current is focused primarily on the few ions. However, the product ions formed by the loss of ammonia or water are not the most specific. Therefore, the use of higher collision energy can yield more specific product ions derived from the dissociation of the ring structure. In this case, however, the sensitivity is reduced, since the ion current is distributed over several product ions. The glucuronides were dissociated by the loss of glucuronide moiety with or without one or two water molecules or ammonia or both, thus providing high sensitivity and specificity. Steroid sulfates were monitored in the negative ion mode using the deprotonated molecule as a precursor ion, and sulfate fragments $[HSO_4]^-$ (at *m/z* 97) and $[SO_3]^-$ (at *m/z* 80) as product ions. The detailed mass spectrometric parameters, retention times, and SRM transitions appear in Table 5.1. The first step in the validation process was to show the valid performance of internal standards spiked into the brain samples (homogenates corresponding to 10 mg of mouse brain). The non-spiked brain samples showed no signals of internal standards, thus showing good specificity for the internal standards. The DLs for d4-allopregnanolone and d3-testosterone glucuronide were 0.9 and 0.01 nmol/L (5.8 and 0.09 ng/g using 10 mg of the mouse whole brain homogenate), respectively. The sample pretreatment was effective as DLs of the internal standards were similar both in standard solutions and brain extracts (Table 5.2). Ten brain extracts from the mouse hippocampus (control samples weighing 26.5 – 39.9 mg) were prepared in order to study the recovery and precision of the method. The average recovery for d4-allopregnanolone was 69.3% (RSD 18.4%, n 10), and for d3-testosterone glucuronide, 94.9% (RSD 11.7%, n 10). These results show that internal standards can be analyzed with high sensitivity and with adequate recovery and repeatability.

Table 5.2. *Validation results of the method: detection limits, low limit of quantitation, linearity, and precision. Abbreviations for compounds appear in Figure 5.1.*

Compound	Limit of detection	Low limit of quantitation		Linearity and range		Precision	
	nmol/L in solution	nmol//L in solution	Precision RSD %, n = 5	Correlation coefficient (r)	Range nmol//L	Intraday RSD %, n = 5	Interday RSD %, n = 9
17-EG	0.006	0.05	7.7	0.9999	0.05-50	2.2	3.6
21-HCG	0.06	0.1	4.0	0.9999	0.1-50	4.8	4.7
TG	0.008	0.05	6.5	0.9999	0.05-20	1.4	3.3
d3-TG	0.01	0.05	18.0	0.9997	0.05-50	2.1	4.4
d3-TG [A]	0.01	ND	ND	0.9997	0.05-5	11.7	ND
21-CORTG	0.015	0.05	11.1	0.9999	0.05-20	7.7	3.0
DHEAG	0.008	0.05	7.3	1.0000	0.05-10	2.2	2.4
HC	0.13	0.5	3.2	0.9987	0.5-100	2.6	6.8
3-THDG	0.01	0.05	5.2	0.9999	0.05-100	3.9	2.0
21-THDG	0.03	0.05	4.5	0.9982	0.05-100	2.5	2.4
PREGG	0.08	0.25	14.5	1.0000	0.25-1000	4.5	2.0
CORT	0.03	0.05	19.7	0.9990	0.05-10	3.0	8.0
IPG	0.018	0.05	9.7	0.9999	0.05-10	2.2	3.7
DHEAS	0.013	0.1	3.6	1.0000	0.1-10	2.3	ND
PREGS	0.032	0.05	2.2	0.9998	0.05-10	3.9	5.6
T	0.026	0.05	11.2	1.0000	0.05-50	3.8	7.0
DHEA	0.75	2.5	8.5	0.9979	2.5-1000	4.3	2.9
E	22	25.0	10.5	0.9995	25-1000	3.4	3.9
THD	0.32	1.0	11.6	1.0000	1-500	1.5	5.3
PROG	0.39	1.0	11.9	0.9999	1-100	2.1	3.7
DHP	2.0	5	13.2	0.9982	5-100	7.9	2.7
PREG	0.87	5.0	6.0	0.9999	5-500	1.0	2.1
IP	2.2	5.0	7.9	0.9994	5-100	2.1	6.6
d4-AP	0.75	1.0	5.2	0.9997	1-1000	1.1	4.7
d4-AP [A]	0.90	ND	ND	0.9915	1-100	18.4	ND

Validation was carried out using either standard solutions or internal standards spiked to homogenates, corresponding 10 mg of whole mouse brain. [A] Measured in the mouse brain homogenate. ND; Not determined.

Determination of the specificity of the method for the analytes with real mouse brain samples was impossible because the real samples contained varying amounts of most of the analytes. The reagent blank samples showed no disturbing peaks in the ion chromatograms of the monitored steroids, or of their glucuronide and sulfate conjugates. Further validation was performed with standard solutions. The calibration curves for each analyte were measured with the internal standard method at concentrations ranging between 10 pmol/L and 1000 nmol/L. The correlation coefficients (r) were better than 0.998 with an accuracy of 80 – 110%, indicating the good quantitative linearity of the method. The calibration remained stable for at least five days. The day-to-day and within-day precisions were studied with standard solutions containing 1 nmol/L of C, T and steroid conjugates, and 100 nmol/L of others. Both the within-day and day-to-day precisions were better than 8.0 % (Table 5.2), indicating good precision. DLs with a signal-to-noise ratio of 10 were 6 – 80 pmol/L (corresponding to 0.014 – 0.19 ng/g of tissue) for steroid glucuronides, 13 – 32 pmol/L (corresponding to 0.013 – 0.063 ng/g of tissue) for sulfates, 26 pmol/L – 2.2 nmol/L for steroids (corresponding to 0.03 – 3.5 ng/g of tissue), except 22 nmol/L for β-estradiol (corresponding to 30 ng/g of tissue) (Table 5.2). The lower limit of quantitation (defined as the lowest concentration with an RSD of less than 20 % in the linear range) were 50 – 250 pmol/L for steroid glucuronides, 50 pmol/L for sulfates and 50 pmol/L – 5 nmol/L for steroids, except 25 nmol/L for β-estradiol (Table 5.2). The standard solutions were stable for at least a week at 4 °C and after three freeze (- 70 °C) and thaw (room temperature) cycles.

Brain extracts. To demonstrate the feasibility of the method developed, we analyzed steroids and their metabolites from mouse hippocampus and hypothalamus (one nicotine-treated mouse and one control mouse from 7-week nicotine treatment experiment). Mice treated with nicotine for seven weeks and control mice treated with water were selected for this study because previous studies have shown that the administration of nicotine raised elevated cerebrocortical and plasma concentrations of pregnenolone, progesterone, and allopregnanolone.[37] However, the aim of this study was develop a method to direct analysis of steroids and their conjugate metabolites in brain tissues, not to study the effects of nicotine in the brain. Six steroid glucuronide conjugates (17-EG, 21-HCG, TG, 21-CORTG, PREGG, and IPG) and four steroids (HC, CORT, PROG, and PREG) were identified and quantified in brain samples. The analytes were identified by comparing of the relative retention times and responses of two SRM pairs of reference standards with those of peaks from brain samples in the capLC-MS/MS analysis. A relative difference of less than 10 % for the ratio of the area of SRM pairs and a relative difference of less than 2 % for the retention times served as criteria for positive identification. Tetrahydroprogesterone (THPROG) has four isomers, i.e., IP (3β,5α), allopregnanolone (3α,5α), 3α,5β-THPROG, and 3β,5β-THPROG, for which respective glucuronide conjugates are not commercially available. We were able to synthesise and characterize only the IP glucuronide,[36] and it was not possible to include the other isomers in the quantitative analysis. Moreover, it is possible that the isomers are not separated from each other in our method, thus the amount of IPG might also include the amounts of other isomeric glucuronides.

The amounts of steroids varied from 1.2 to 58 ng/g, and the amounts of steroid glucuronides varied from 0.2 to 3.7 ng/g in the mouse hypothalamus and hippocampus (Table 5.3). In earlier studies, the amounts of neurosteroids have ranged from 0.06 to 20 ng/g in untreated whole rat brains,[10,24,26,30] although the amounts for separated brain areas of mouse brain have not been yet published. This is the first time that steroid glucuronides have been quantified from brain samples. The SRM chromatograms of steroids and their glucuronide conjugates from a standard solution, a nicotine-treated mouse hippocampus sample, and a reagent blank sample appear in Figure 5.3. After comparing of the amounts (mol/g) of native steroids and their glucuronide conjugates, we suggest that glucuronidation might be an important metabolic pathway for some steroids in the mouse brain. For example, the molar ratio of 21-HCG/HC was 19 – 128 %, that of 21-CORTG/CORT was 1 – 5 %, and that of PREGG/PREG was 0.2 – 10 % (semi quantitative estimation as the concentrations were between LD and LQ). Steroid sulfates were not detected in the samples, which is in agreement with other direct methods. The DLs of sulfate conjugates in our method should be low enough for the detection when compared with the amounts of sulfate conjugates reported with indirect methods. Some researchers have suggested that steroid sulfates, detected with indirect methods using hydrolysis and derivatization, are actually other conjugates, such as steroid glucuronides[10] or lipid conjugates.[11] Autoxidation of cholesterol during insufficient sample preparation and hydrolysis has also been presented as a possible source of steroid sulphates[12].High brain steroid sulphatase (STS) activity can be another explanation for the absence of steroid sulfates in the rodent brain.[38]

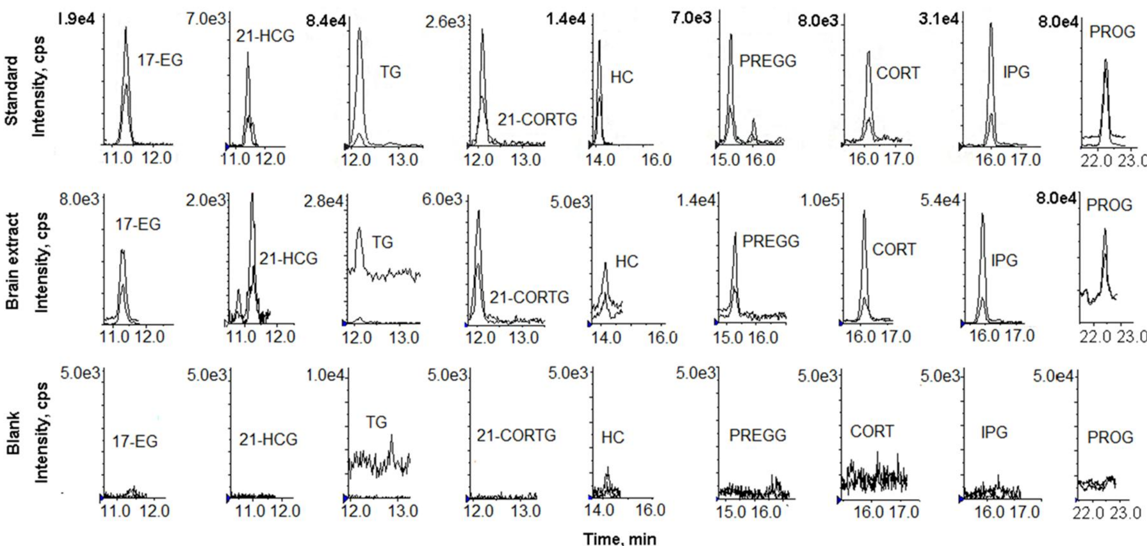

Figure 5.3. *SRM chromatograms of steroids and their glucuronide conjugates from a standard solution (upper), a nicotine-treated mouse hippocampus sample (middle), and a reagent blank sample (lower). Each conjugate was analyzed using two SRM pairs (see Table 5.1), and only compounds detected from mouse hippocampus are shown.*

Table 5.3. *Quantitative results of steroids and their conjugates from brain tissues of nicotine-treated or control mice (7-week treatment). Abbreviations for compounds appear in Figure 5.1.*

Compound	Mouse hippocampus		Mouse hypothalamus	
	Nicotine-treated ng/g	Control ng/g	Nicotine-treated ng/g	Control ng/g
17-EG	0.6	1.2	1.2	1.0
21-HCG	1.5	3.4	3.4	1.5
TG	0.2	< LQ	< DL	< DL
21-CORTG	0.5	0.9	1.2	0.7
DHEAG	< DL	< DL	< DL	< DL
HC	1.2	4.3	10	7.8
3-THDG	< DL	< DL	< DL	< DL
21-THDG	< LQ	< LQ	< LQ	< DL
PREGG	1.7	0.4	< LQ	< DL
CORT	22	11	58	27
IPG	3.7	2.8	1.8	< LQ
DHEAS	< DL	< DL	< DL	< DL
PREGS	< DL	< DL	< DL	< DL
DHEA	< LQ	< LQ	< LQ	< LQ
E	< DL	< DL	< DL	< DL
THD	< DL	< DL	< DL	< DL
PROG	18	21	< QL	47
DHP	< LQ	< LQ	< LQ	< LQ
PREG	< LQ	< LQ	13	< LQ
IP	< DL	< DL	< DL	< DL

DL; detection limit, LQ; low limit of quantitation. The concentration of testosterone is not reported because of background interferences at low concentration.

5.4 Conclusions

The method developed here provides an important new tool for neuropharmacological studies. The method developed provides for the first time a direct quantitative determination of intact steroid glucuronides and sulfates without hydrolysis, and therefore creates the possibility to study the role of steroid glucuronidation and sulphation in the brain. The new method combines a relatively simple sample preparation and a capillary LC-ESI-MS/MS method for the identification and quantification of 21 steroids, steroid glucuronides and sulfates in mouse brain tissues. High sensitivity was obtained for steroid glucuronides and sulfates, which is from ten to some hundreds of times better than that obtained with previously reported methods for non-neurosteroid glucuronide conjugates. For free steroids, sensitivity varied more, depending on the structure of the steroid, but the DLs obtained were low enough for the analysis of real samples. Derivatization makes it possible to achieve lower detection limits for intact steroids, but derivatization is restricted to steroids having a certain functional group, the pretreatment procedure is more complicated and a possible source of errors, and the use of the same pretreatment and analytical procedure for steroids, glucuronides, and sulfates is impossible with derivatization. The method developed is also applicable for intact brain microdialysates with no modification, but further studies are needed to validate this method for microdialysis samples.

Several steroids and steroid glucuronides were identified and quantified from the mouse hippocampus and hypothalamus at a level of nmol/g or less, and the measured concentrations were in a rational range when compared to previously reported results of neurosteroids from a whole rat brain. By comparing the amounts (mol/g) of native steroids and their glucuronide conjugates, we suggest that glucuronidation might be an important metabolic pathway for some steroids in the mouse brain. The role of steroid glucuronidation in the brain still remains unknown, however. Further studies are needed to develop analytical methods to measure concentrations of steroids and their conjugates from plasma, urine, and microdialysis samples to be able to investigate the origin and function of steroid glucuronidation in brain.

5.5 Acknowledgement

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5.6 References

[1] Mellon, S. H.; Griffin, L. D. *Trends Endocrinol. Met.* **2002**, *13*, 35-43.
[2] Stoffel-Wagner, B. *Ann N. Y. Acad. Sci.* **2003**, *1007*, 64-78.
[3] Belelli, D.; Lambert, J. L. *Nat. Rev. Neurosci.* **2005**, *6***,** 565-575.
[4] Do Rego, J.; Seong, J.; Burel, D.; Leprince, J.; Luu-The, V.; Tsutsui, K.; Tonon, C-M.; Pelletier, G.; Vaudry, H. *Front. Neuroendocrinol.* **2009**, 30, 259-301.
[5] Stoffel-Wagner, B. *Eur. J. Endocrinol.* **2001**, *45*, 669-679.
[6] Mensah-Nyagan, A.; Do-Rego, JL.; Beaujean, D.; Luu-The, V.; Pelletier, G.; Vaudry, H. *Pharmacol. Rev.* **1999***, 51, 63-81.*
[7] Agís-Balboa, R.; Pinna, G.; Zhubi, A.; Maloku, E.; Veldic, M.; Costa, E.; Guidotti, A. *Proc. Natl. Acad. Sci. USA* **2006**, *103,* 14602-14607.
[8] Corpechot, C.; Robel, P.; Axelson, M.; Sjövall, J.; Baulieu, E. E. *Proc. Natl. Acad. Sci. USA* **1981,** *78,* 4704-4707.
[9] Corpechot, C.; Synguelakis, M.; Talha, S.; Axelson, M.; Sjövall, J.; Vihko, R.; Baulie, E. E.; Robel, P. *Brain Res.* **1983**, *270,* 119-125.
[10] Liu, S.; Sjovall, J.; Griffiths, W. J. *Anal. Chem.* **2003,** 75*(21),* 5835-5846.
[11] Schumacher, M.; Liere, P., Akwa, Y.; Rajkowski, K. Griffits, W.; Bodin, K.; Sjövall, J.; Baulieu, E.E. *Neurochem. Int.* **2008**, *52*, 522-540.
[12] Liere, P.; Pianos, A.; Eyechenne, B.; Cambourg, A.; Bodin, K.; Griffiths, W.; Schumacher, M.; Baulieu, E.E.; Sjövall, J. *J. Lipid Res.* **2009**, *50*, 2430-2444.
[13] King, C. D.; Rios, G. R.; Assouline, J. A.; Tephly, T. R. *Arch. Biochem. Biophys.* **1999**, *365,* 156-162.
[14] Suleman, F. G.; Abid, A.; Gradinaru, D.; Daval, J. L.; Magdalou, J.; Minn, A. *Arch. Biochem. Biophys.* **1998,** *358*, 63-67.
[15] Jedlitschky, G.; Cassidy, A. J.; Sales, M.; Pratt, N.; Burchell, B. *Biochem. J.* **1999,** *340,* 837-843.
[16] Belanger, G., Barbier, O., Hum, D., Belanger, *Eur. J. Biochem*. **1999**, *260*, 701-708.
[17] Kallonen, S.E.; Tammimäki, A.; Piepponen, P.; Raattamaa, H.; Ketola, R.A.; Kostiainen, R. *Anal. Chim. Acta* **2009**, *651*, 69-74.
[18] Uutela, P.; Karhu, L.; Piepponen, P.; Käenmäki, M.; Ketola, R.A.; Kostiainen, R. *Anal. Chem.* **2009**, *81*, 427-434.
[19] Uutela, P.; Reinilä, R.; Harju, K.; Piepponen, P.; Ketola, R.A.; Kostiainen, R. *Anal. Chem.* **2009**, *81*, 8417-8425.
[20] Borts, D.; Bowers, L. J. *J. Mass Spectrom.* **2000**, *35*, 50-61.
[21] Yang, Y.; Lee, J.; Choi, M.; Chung B. *Biomed. Chromatogr.* **2003***, 17,* 219-225.
[22] Hintikka, L.:, Kuuranne, T.;, Leinonen, A.; Thevis, M.;, Schanzer, W.; Halket, J.; Cowan, D,; Grosse, J.; Hemmersbach, P.; Nielen, M.; Kostiainen, R. *J. Mass Spectrom.* **2008,** *43(7),* 965-973.
[23] Corpéchot, C.; Young, J.; Calvel, M.; Wehrey, C.; Veltz, J. N.; Touyer, G.; Mouren, M.; Prasad, V. V. K.; Banner, C.; Sjövall, J.; Baulieu E. E.; Robel, P. *Endocrinology* **1993**, *133*, 1003-1009.
[24] Liere, P.; Akwa, Y.; Weill-Engerer, S.; Eychenne, B.; Pianos, A.; Robel, P.; Sjövall, J.; Schumacher, M.; Baulieu., E. E. *J. Chromatogr. B* **2000**, *739*, 301-312.

[25] Vallée, M.; Rivera, J. D.; Koob, G. F.; Purdy R. H.; Fitzgerald., R. L. *Anal. Biochem.* **2000**, *287*, 153-166.
[26] Ebner, M. J.; Corol, D. I.; Havlíková, H.; Honour, J. W.; and Fry, J. P. *Endocrinology* ***2006**, 147,* 179-190.
[27] Griffiths, W.; Liu, S.; Avelius, G.; Sjöval, J. *Rapid Commun. Mass Spectrom.* **2003**, *17,* 924-935.
[28] Griffiths, W.; Alvelius, G.; Liu, S.; Sjöval, J. *Eur. J. Mass Spectrom.* **2004**, *10*, 63-88.
[29] Higashi, T.; Takido, N.; Shimada, K. *Steroids* **2005**, *70*, 1-11.
[30] Higashi, T.; Nagahama, A.; Otomi, N.; Shimada, K. *J. Chromatogr. B* **2007,** *848*, 188-199.
[31] Higashi, T.; Ninomiya, Y.; Iwaki, N.; Yamauchi, A.; Takayma, N.; Shimada, K. S*teroids* **2006,** *71*, 609-617.
[32] Higashi, T.; Daifu, Y.; Shimada, K. *Steroids* **2001**, *66*, 865-874.
[33] Kuuranne, T.; Aitio, O.; Vahermo, M.; Elovaara, E.; Kostiainen, R. *Bioconjugate Chem.* **2002**, *13*, 194-199.
[34] Kuuranne, T.; Kurkela, M.; Thevis, M.; Schänzer, W.; Finel, M.; Kostiainen, R. *Drug Metab. Dispos.* **2003**, *31*, 1117-1124.
[35] Jäntti, S.; Kiriazis, A.; Reinilä, R.; Kostiainen, R.; Ketola, R. A. *Steroids* **2007**, *72,* 287-296.
[36] Kuuranne, T.; Vahermo, M.; Leinonen, A.; Kostiainen, R. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 722-730.
[37] Concas, A.; Sogliano, C.; Porcu, P.; Marra, C.; Brundu, A.; Biggio, G. *Psychopharmacol.* **2006**, *186*, 281-292.
[38] Steckelbroek, S.; Nassen, A.; Ugele, B.; Ludwig, M.; Watzaka, M.; Reissinger, A.; Clusmann, H., Lutjohann, D.; Siekmann, L.; Klingmuller, D.; Volkmar, H. *J. Neurochem.* **2004**, *89*, 403-417.

6 Determination of Steroid and Steroid Glucuronide Profiles in Pregnancy Urine Samples by Liquid Chromatography-Electrospray Ionisation-Tandem Mass Spectrometry

An ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS) method was developed for the analysis of steroids and their glucuronides in urine samples. The method provides high sensitivity and fast analysis, as both steroids and their glucuronides can be analyzed directly without hydrolysis or complex sample preparation. The method was applied in profiling of targeted and nontargeted steroids and steroid glucuronides during pregnancy. The concentrations of 11 of 27 targeted steroids and steroid glucuronides and the concentrations of 25 nontargeted steroid glucuronides increased about 10–400 fold during the pregnancy. The concentrations of most of these 36 compounds began to increase in the first days of the pregnancy, increased gradually during the pregnancy, achieved a maximum in late pregnancy, and decreased sharply after delivery. Exceptionally, the concentrations of allopregnanolone and 17-hydroxypregnenolone started to increase later than those of the other steroids. Moreover, the concentrations of E2 glucuronides began to decrease one week before the delivery, in contrast to most of the steroids and steroid glucuronides, whose concentrations dropped sharply during the delivery. Concentrations of 34 compounds decreased noticeably when the subject was on sick leave owing a series of painful contractions. The results suggest that steroids and especially steroid glucuronides may provide a valuable diagnostic tool to follow the course of pregnancy.

6.1 Introduction

Steroids are synthesized mainly by endocrine glands, released into the blood circulation, and metabolized typically by reduction, oxidation, and hydrolysis reactions. Hydroxylated metabolites are often metabolized further via phase II metabolic reactions to conjugates, which are more polar, have lower affinity for plasma proteins, and are secreted more easily to urine. Glucuronidation takes place mainly in the liver, although some metabolic enzymes are present in other organs, including the kidney, intestines, lung, prostate, gut, and brain [1-4]. Glucuronidation is one of the most common pathways of conjugative metabolism [1,5-6], and is a major conjugation route for steroid in human.

Endogenous estrogens and progestins have an important role in controlling and maintaining the course of normal pregnancy [7]. The concentrations of several steroids have been shown to change significantly during pregnancy and postpartum [8-15]. The role of neuroactive steroids, which are modulators of several neurotransmitter receptors [16-18], is less well known [19]. The important neuroactive steroid allopregnanolone (AP, $3\alpha,5\alpha$-THPROG) and its isomers pregnanolone (P), isopregnanolone (IP), and epiAP are formed from progesterone (PROG) in a two-step reduction reaction catalyzed by $5\alpha/5\beta$-reductase and $3\alpha/3\beta$-hydroxysteroid oxidoreductase [15]. Levels of neuroactive steroids

are relevant to mood during pregnancy and postpartum. For example, low levels of AP in plasma or cerebrospinal fluid have been linked to prenatal and postpartum depression [20]. Decrease in the levels of AP could also trigger the production of oxytocin resulting in a rapid delivery [21-22]. The role of steroids during pregnancy has recently been reviewed by Hill et al. [19].

Several methods are applied in the analysis of steroids, the most common of these being radioimmunoassay (RIA), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). RIA, the traditional method for steroid analysis, is applied for the determination of PROG, AP, and 5α-THDOC during pregnancy in blood [23-24]. However, the suitability of RIA for the analysis of complicated samples, such as pregnancy urine, is questionable owing the possibility of cross reactions. GC-MS, which provides high sensitivity and selectivity, is widely used for the analysis of steroids in biological samples [25-27] and is also applied to the analysis of steroids during pregnancy [15,19,28]. Typically the steroids are derivatized before GC-MS analysis. Evans et al. [28] showed the concentrations of five metabolites of PROG to increase significantly between weeks 10 and 36 of pregnancy. Glucuronide and sulfate conjugates of steroids have also been analyzed by GC-MS after hydrolysis. Meng and co-workers [29-30] studied progesterone metabolites and their sulfate and glucuronide conjugates in serum and urine of pregnant women by GC-MS. Recently Hill et al. [15] analyzed 40 steroids and 26 conjugates in blood samples after chemical hydrolysis by GC-MS, using four data points during the pregnancy. They observed that the concentrations of conjugated steroids were 5 - 100 times those of nonconjugated steroids and suggested that the balances between free steroids and their conjugates may be crucial for regulation of the biological activity of these compounds and consequently for the sustaining of pregnancy.

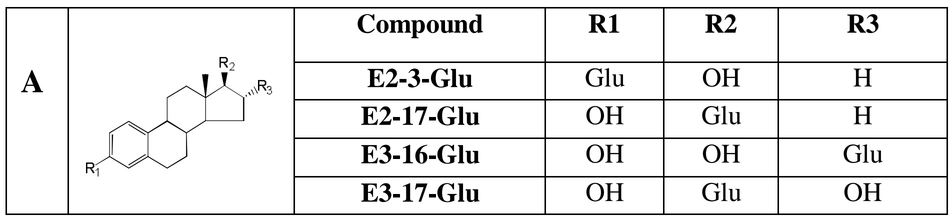
The problem with GC-MS is the hydrolysis step, which precludes the direct analysis of steroid conjugates and complicates sample pretreatment. In addition, methods containing a hydrolysis step do not provide identification of the conjugates with absolute certainty, even when specific enzymes such as β-glucuronidase or sulfatase are utilized.

LC-MS, which is a fast, easy, and highly sensitive method, is increasingly being used for the analysis of steroids [31-32]. Most of the quantitative LC-MS methods for the analysis of steroid conjugates nevertheless employ a hydrolysis step, and thus far only a few methods have been published for the quantification of intact steroid conjugates [33-38]. This is probably because of the limited availability of commercial steroid conjugate standards. Furthermore, the current methods are based on the use of HPLC- or capillary LC-MS/MS. Although MS/MS provides high selectivity, the chromatographic resolution may be limited with HPLC or capillary LC for the separation of isomers of steroid glucuronides. In view of the lack of earlier determinations of intact steroid glucuronides during pregnancy, we undertook to evaluate the feasibility of ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for profiling 27 steroids and their glucuronides in urine during pregnancy. The method combines high chromatographic resolution of UPLC and high specificity and sensitivity of MS/MS. In addition we used UPLC- high resolution mass spectrometry for the identification of nontargeted steroid glucuronides, of which concentrations were increased during the pregnancy. The period from 10 days after gestation to 25 days postpartum was investigated.

6.2 Experimental

Reagents and standards. Cortisol (HC, 4-pregnen-11ß,17α,21-triol-3,20-dione), dehydroepiandrosterone (DHEA, 5-androsten-3ß-ol-17-one), 5α-dihydroprogesterone (5α-DHP, 5α-pregnan-3,20-dione), isopregnanolone (IP, 5α-pregnan-3ß-ol-20-one), pregnenolone (PREG, 5-pregnen-3ß-ol-20-one), progesterone (PROG, 4-pregnane-3,20-one), testosterone (TES, 4-androsten-17ß-ol-3-one), dehydroepiandrosterone 3-glucuronide (DHEA-Glu), estradiol 3-glucuronide (E2-3-Glu, 1,3,5(10)-estratriene-3,17β-diol 3-glucuronide), estradiol 17-glucuronide (E2-17-Glu, 1,3,5(10)-estratriene-3,17β-diol 17-glucuronide), 11-deoxycortisone (11-DC, 4-pregnanen-17,21-diol-3,20-dione), 17-hydroxypregnenolone (17-OH-PREG, 5-pregnen-3β,17α-diol-20-one), aldosterone (AL, 4-pregnen-11β,17α-diol-3,18,20-trione), allopregnanolone (AP, 5α-pregnan-3α-ol-20-one), pregnanolone (P, 5β-pregnan-3α-ol-20-one), cortisone (CS, 4-pregnen-17α,21-diol-3,11,20-trione), estriol 16-glucuronide (E3-16-Glu, 1,3,5(10)-estratriene-3,16α,17β-triol, 16-glucuronide), estriol 17-glucuronide (E3-17-Glu, 1,3,5(10)-estratriene-3,16α,17β-triol, 17-glucuronide), 5α-tetrahydrodeoxycorticosterone (5α-THDOC, 5α-pregnan-3α,21-diol-20-one) and dihydrotestosterone (DT, 5α-androstan-17ß-ol-3-one) standards were supplied from Sigma-Aldrich (St. Louis, MO, USA); corticosterone (CORT, 4-pregnen-11ß,21-diol-3,20-dione) was supplied from Fluka Chemie GmbH (Buchs, Switzerland); testosterone glucuronide (TES-Glu), pregnenolone glucuronide (PREG-Glu) and androstenedione (AN, 4-androstene-3,17-dione) were from Steraloids Inc. (Newport, RI, USA), and d3-nandrolone (d3-5α-N, d3-17ß-hydroxy-5α-estra-4-ene-3-one) was from Cambridge Isotopes Laboratories (Andover, MA, USA). Isopregnanolone glucuronide (IP-Glu), 5α-tetrahydrodeoxycorticosterone 3- and 21-glucuronides (5α-THDOC-3- and 21-Glu), and corticosterone 21-glucuronide (CORT-21-Glu) were synthesized, purified, and characterized earlier in our laboratory [39]; and d5-5α-androstane-3α-ol-17β-glucuronide (d5-A-Glu) was obtained from the Institute of Biochemistry, Cologne, Germany. The structures of the studied steroids and their metabolites are presented in Table 6.1. Methoxyamine hydrochloride (MOX) from Pierce (Fisher Scientific UK Ltd, Loughborough, UK) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and chlorotrimethylsilane from Sigma-Aldrich (St. Louis, MO, USA) were used as derivatization reagents. All reagents were reagent grade, and solvents were HPLC grade.

Sample collection and purification of urine samples. Thirteen morning urine samples were collected from healthy volunteer 1 during pregnancy, one sample was collected on delivery day, and three samples were taken after delivery (Figure 6.1). The delivery was performed as a C-section and an overnight (10 hours) urine sample from the delivery day was collected from a catheter. One morning urine sample was collected from an earlier pregnancy of volunteer 1 at week 35 (delivery at week 35) and one sample was collected from healthy volunteer 2 in week 37 of her pregnancy (delivery at week 40). These two samples were used to compare steroid profiles for three different pregnancies. One control (nonpregnancy) urine sample was collected form volunteer 1 (day 4 of menstrual cycle). Samples were frozen instantaneously after collection and thawed before analysis. Internal standards (9 µL mixture of 1.5 µg mL-1 d5-A-Glu and 15 µg mL-1 d3-5α-N) were added to 3 mL of urine before analysis.



A	Compound	R1	R2	R3
	E2-3-Glu	Glu	OH	H
	E2-17-Glu	OH	Glu	H
	E3-16-Glu	OH	OH	Glu
	E3-17-Glu	OH	Glu	OH

B	Compound	R1	R2	R3
	DHEA	OH	=O	-
	DHEA-Glu	Glu	=O	-
	17-OH-PREG	OH	$COCH_3$	OH
	PREG	OH	$COCH_3$	H
	PREG-Glu	Glu	$COCH_3$	H

C	Compound	R1	R2	R3	R4
	AL	OH	HC=O	CH_2OH	H
	CORT	OH	CH_3	CH_2OH	H
	CORT-21-Glu	OH	CH_3	CH_2Glu	H
	CS	=O	CH_3	CH_2OH	OH
	11-DC	H	CH_3	C_2H_5	OH
	HC	OH	CH_3	CH_2OH	OH
	PROG	H	CH_3	CH_3	H

D	Compound	R1	R2	R3
	AP	OH (α)	HCO	**H (α)**
	5α-DHP	=O	HCO	**H (α)**
	DT	=O	OH	**H (α)**
	IP	OH (β)	HCO	**H (α)**
	IP-Glu	Glu (β)	HCO	**H (α)**
	5α-THDOC	OH (α)	$COCH_2OH$	**H (α)**
	5α-THDOC-3-Glu	Glu (α)	$COCH_2OH$	**H (α)**
	5α-THDOC-21-Glu	OH (α)	$COCH_2Glu$	**H (α)**

E	Compound	R1
	AN	=O
	TES	OH
	TES-Glu	Glu

Structure of Glu

Table 6.1. *Structures of studied steroids and their glucuronide conjugates.*

The samples were purified by solid phase extraction (SPE) (Oasis HLB 60 mg, 3cc, Waters, MA, USA). Cartridges were conditioned with methanol (3 mL) and then with water (3 mL). A urine sample (3 mL) was loaded and the cartridge was washed with 10% of methanol (3 mL). Steroids and their respective conjugates were eluted from the cartridge with acetonitrile (3 mL), evaporated with nitrogen, reconstituted with 25 µL of 50% methanol, and diluted with 275 µL of water. Spiked artificial urine samples [40] used in the method development, and they were purified similarly to the authentic urine samples. Standard were prepared using concentration of 10 ng mL-1 for all steroid glucuronides and steroids with 3-one-4-ene structure (testosterone and class C in Table 6.1), and 50 ng mL-1 for others, respectively. The concentrations of steroids and steroid glucuronides were normalized using apparent densities of urea samples measured by DMA 58 density meter (Anton PAAR GbmH, Österreich, Austria).

Ultra performance liquid chromatography-tandem mass spectrometry.The urine samples were analyzed by ultra performance liquid chromatography (UPLC-MS/MS). The LC was an Acquity UPLC, and a BEH Shield RP18 (1.0x150 mm, 1.7 µm) (Waters, MA, USA) was used as analytical column. The mobile phase A was 50 mM NH_4Ac (pH 4.5 with acetic acid) / water (1:9, v/v) and the mobile phase B was 50 mM NH_4Ac (pH 4.5 with acetic acid) / methanol / acetonitrile (1:3:6, v/v/v). The gradient program was from 5% to 25% B (1 min), to 60% B (25 min), to 100% B (26 min), and back to 5% B (27 min). The run time was 32 min including the equilibrium time of 5 min. The flow rate was 0.13 mL min^{-1} and the temperature of the column was 50 ºC. The thermostat of the sampler was set to 4 ºC and the injection volume was 10 µL. Flow from the UPLC was split before the MS so that the flow rate to the MS was about 30 µL min^{-1}.

The mass spectrometer used in both the targeted and nontargeted methods was an API 3000 triple quadrupole instrument (AB Sciex, Toronto, ON, Canada) with a turbo ion spray source operated at 5500 V. Purified air (Atlas Copco, Belgium) was used as a nebulizing gas, and nitrogen generated by a nitrogen generator (Generator 75-72, Whatman, Clifton, NJ, USA) was used as curtain and collision gases. Temperature of the ion source was 150 ºC. Targeted steroids and their glucuronide metabolites were analyzed with scheduled selected reaction monitoring (SRM) with dwell times of 130-400 ms (detection window of 30 s, target time of 0.6 s). The declustering potentials and the collision energies were optimized separately for each precursor–product ion transition. Unknown steroid glucuronides were screened using 2-3 SRMs for precursor ions *m/z* 512, 514, 528, and 530. Dwell time in these runs was 30 ms. Internal standard method was used for the quantification. The SRM conditions are presented in Table 6.A.1.

Accurate mass measurements. The accurate mass measurements were performed by UPLC–high resolution mass spectrometry (HRMS). The UPLC and the chromatographic conditions were the same as used in the UPLC-MS/MS method described above. The mass spectrometer was an LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Nanomate Triversa nano-electrospray (Advion, Ithaca, NY, USA), which was operated in positive ion mode with spray voltage of 1.7 kV and capillary temperature of 200 ºC. The data were collected in centroid mode at a mass range of m/z 250–600, and the instrument was calibrated using external calibration. The mass resolution was 60000 at m/z 400.

Gas chromatography - mass spectrometry. Three samples from late pregnancy from volunteer 1 were pooled and fractionated by UPLC with use of the same chromatographic conditions as in the UPLC-MS/MS method described above. The most important compounds were collected to a Nanomate Eppendorf twin.tec 96 tray (95 fractions in several segments between 5-17 min and fraction width of 5 s), hydrolyzed and derivatized before GC-MS analysis. The samples were evaporated to dryness with nitrogen, 1 mL of chlorotrimethylsilane was added, and incubation was carried out at 70 ºC for 60 min. Thereafter the samples were dried under a gentle flow of nitrogen before two-step derivatization. In the first step, the steroids were converted to their methoxime (MEOX) derivatives by addition of 25 µL of MOX reagent and incubation of the samples at 45 ºC for 60 min. In the second step, TMS derivatives were produced by adding 100 µL of MSTFA reagent and incubating the samples at 45 ºC for 60 min. The samples were injected to GC as such. Saturated n-alkanes up to C30 from Supelco (Sigma-Aldrich) were used to calculate linear retention indices. The NIST 2008 Mass Spectral Library was used as spectral library. Samples were analyzed with an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector and an Agilent GC Sampler 120 (Santa Clara CA, USA). A 1.5 m × 0.53 mm I.D deactivated retention gap (Santa Clara CA, USA) was connected to a 30 m × 0.25 mm I.D. 5% phenyl / 95% dimethylpolysiloxane column with 0.25 µm film thickness (ValcoBond VB-5, Vici Valco, Schenkon, Switzerland). Helium was used as a carrier gas at flow of 1.2 mL min-1. The injected sample volume was 1 µL and the injector (250 °C) was operated with a 1 min splitless period. The run was initiated with a 2 min isothermal period at 50 ºC, after which the oven temperature was raised to 330 ºC at 7 °C min-1 and held at 330 ºC for 5 min. The interface was maintained at 330 ºC. The compounds were ionized by electron ionization at 70 eV. The ion source temperature was 230 ºC. The recorded mass range was m/z 50–600.

Visualisation of steroid profile. The heatmap (Figure 6.1) visualizing the steroid profile during pregnancy was constructed using R version 2.15.2 and gplots library. The data for each compound in the heatmap were normalized so that the minimum and maximum values across all samples were 0 and 100, respectively.

Table 6.A.1. *Operating parameters for selected reaction monitoring of steroids and steroid glucuronides.*

Compound	*Internal standard*	*SRM*	*Precursor ion*	*Product ion*	*DP*[1] *(V)*	*CE*[2] *(V)*
E2-3-Glu	D4-5α-NG	466→ 141	$[M+NH_4]^+$	$[Glu+H-2H_2O]^+$	30	35
		466→ 159	$[M+NH_4]^+$	$[Glu+H-H_2O]^+$	30	33
E2-17-Glu	D4-5α-NG	466→ 273	$[M+NH_4]^+$	$[M+H-Glu]^+$	30	20
		466→ 194	$[M+NH_4]^+$	unknown	30	20
E3-16/17-Glu	D4-5α-NG	482→ 271	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	22
		482→ 289	$[M+NH_4]^+$	$[M+H-Glu]^+$	20	23
DHEA	D4-NA	306→ 271	$[M+NH_4]^+$	$[M+H-H_2O]^+$	27	15
		306→ 253	$[M+NH_4]^+$	$[M+H-2H_2O]^+$	27	18
DHEA-Glu	D4-5α-NG	482→ 253	$[M+NH_4]^+$	$[M+H-Glu-2H_2O]^+$	30	40
		482→ 97	$[M+NH_4]^+$	From the A-ring	30	40
17-OH-PREG	D4-NA	350→ 315	$[M+NH_4]^+$	$[M+H-H_2O]^+$	22	20
		350→ 297	$[M+NH_4]^+$	$[M+H-2H_2O]^+$	22	15
PREG	D4-NA	334→ 317	$[M+NH_4]^+$	$[M+H]^+$	25	25
		317→ 299	$[M+H]^+$	$[M+H-H_2O]^+$	80	15
PREG-Glu	D4-5α-NG	510→ 299	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	25	20
		510→ 281	$[M+NH_4]^+$	$[M+H-Glu-2H_2O]^+$	25	40
AL	D4-NT	361→ 343	$[M+H]^+$	$[M+H-H_2O]^+$	35	25
		361→ 315	$[M+H]^+$	$[M+H-H_2-CO_2]^+$	25	25
CORT	D4-NT	347→ 329	$[M+H]^+$	$[M+H-H_2O]^+$	25	25
		347→ 109	$[M+H]^+$	from the A-ring	25	30
CORT-21-Glu	D4-5α-NG	523→ 329	$[M+H]^+$	$[M+H-Glu-2H_2O]^+$	35	30
		540→ 329	$[M+NH_4]^+$	$[M+H-Glu-2H_2O]^+$	35	30
CS	D4-NT	361→ 163	$[M+H]^+$	Unknown	35	37
		361→ 121	$[M+H]^+$	From the A-ring	35	50
11-DC	D4-NT	347→ 109	$[M+H]^+$	from the A-ring	35	38
		347→ 97	$[M+H]^+$	from the A-ring	35	33
HC	D4-NT	363→121	$[M+H]^+$	From the A-ring	20	20
		363→327	$[M+H]^+$	$[M+H-2H_2O]^+$	20	20
PROG	D4-NA	315→ 97	$[M+H]^+$	from the A-ring	50	35
		315→ 109	$[M+H]^+$	from the A-ring	50	35
AP	D4-NT	336→ 301	$[M+NH_4]^+$	$[M+H-H_2O]^+$	60	15
		336→ 257	$[M+NH_4]^+$	Unknown	60	15
5α-DHP	D4-NA	334→ 317	$[M+NH_4]^+$	$[M+H]^+$	25	15
		317→ 299	$[M+H]^+$	$[M+H-H_2O]^+$	80	15
DT	D4-NA	308→ 255	$[M+NH_4]^+$	$[M+H-2H_2O]^+$	60	25
		291→ 255	$[M+H]^+$	$[M+H-2H_2O]^+$	25	20
IP	D4-NA	336→ 301	$[M+NH_4]^+$	$[M+H-H_2O]^+$	60	15
		336→ 257	$[M+NH_4]^+$	$[M+H-COCHOH-H_2]^+$	60	15
IP-Glu	D4-5α-NG	512→ 301	$[M+NH_4]^+$	$[M+H-H_2O]^+$	35	20
		512→ 141	$[M+NH_4]^+$	$[Glu+H-2H_2O]^+$	35	35
5α-THDOC	D4-NA	352→ 335	$[M+NH_4]^+$	$[M+H]^+$	30	15
		352→ 317	$[M+NH_4]^+$	$[M+H-H_2O]^+$	30	17
5α-THDOC-3-Glu	D4-5α-NG	528→335	$[M+NH_4]^+$	$[M+H-Glu]^+$	20	20
		528→317	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	20
5α-THDOC-21-Glu	D4-5α-NG	528→335	$[M+NH_4]^+$	$[M+H-Glu]^+$	20	20
		528→317	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	20	20
AN	D4-NT	304→ 109	$[M+NH_4]^+$	from the A-ring	15	36
		304→ 287	$[M+NH_4]^+$	$[M+H]^+$	15	10
TES	D4-NT	289→ 109	$[M+H]^+$	from the A-ring	30	40
		289→ 97	$[M+H]^+$	from the A-ring	30	35
TES-Glu	D4-5α-NG	482→ 97	$[M+NH_4]^+$	From the A-ring	25	35
		482→ 271	$[M+NH_4]^+$	$[M+H-Glu-H_2O]^+$	25	25

Nontargeted Steroid glucuronides						
E3-3-Glu	D5-A-Glu	482→ 271	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}H_2O]^+$	20	22
		482→ 289	$[M+NH_4]^+$	$[M+H\text{-}Glu]^+$	20	23
$C_{27}H_{42}O_8$ including P-Glu and AP-Glu	D5-A-Glu	512→ 319	$[M+NH_4]^+$	$[M+H\text{-}Glu]^+$	20	20
		512→ 301	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}H_2O]^+$	20	20
		512→ 283	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}2H_2O]^+$	20	20
$C_{27}H_{44}O_8$	D5-A-Glu	514→ 303	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}H_2O]^+$	20	20
		514→ 285	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}2H_2O]^+$	20	20
$C_{27}H_{42}O_9$	D5-A-Glu	528→ 335	$[M+NH_4]^+$	$[M+H\text{-}Glu]^+$	20	20
		528→ 299	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}2H_2O]^+$	20	20
$C_{27}H_{44}O_9$	D5-A-Glu	530→ 319	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}H_2O]^+$	20	20
		530→ 301	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}2H_2O]^+$	20	20
		530→ 281	$[M+NH_4]^+$	$[M+H\text{-}Glu\text{-}3H_2O]^+$	20	20
Internal standards						
D5-A-Glu	-	474→ 263	$[M+H]^+$	$[M+H\text{-}Glu\text{-}H_2O]^+$	25	20
D4-NA	-	298→ 245	$[M+NH_4]^+$	$[M+H\text{-}2H_2O]^+$	35	25
D3-5α-N	-	295→ 278	$[M+NH_4]^+$	$[M+H]^+$	17	15

[1] DP Declustering potential, [2] CE Collision energy.

6.3 Results and discussion

Analysis of targeted steroids and steroid glucuronides. The changes in concentrations of sixteen targeted steroids and eleven targeted steroid glucuronides (Table 6.1, Figure 6.1) in morning urine samples collected during pregnancy and in four samples collected after delivery were determined by UPLC-MS/MS with selected reaction monitoring (SRM). The glucuronides were analyzed by direct method without a hydrolysis step. The direct method provides more reliable analysis of glucuronides than the indirect method employing a hydrolysis step, since the hydrolysis of conjugates may be unspecific even if enzymatic hydrolysis is used. Moreover, separation of positional glucuronide isomers is not possible after hydrolysis. Except for E3-16-Glu and E3-17-Glu, all the analytes, including α/β and positional isomers, were well separated by our UPLC method (Figure 6.2). The precursor and product ions selected for SRM were ions that provided good sensitivity and as good selectivity as possible (for details see Table 6.A.1). For 17-OH PREG, PREG, 5-alpha-DHP, DT, IP product ions formed by the losses of ammonia and water were chosen for SRM that provides provide maximum sensitivity but compromise the selectivity. However, this was necessary in order to achieve high enough sensitivity, as the ionization efficiencies of these steroids are relatively low for the reasons discussed below. Identification of the targeted steroids and their glucuronides was done by comparison of the relative retention times and relative abundances of two SRM pairs of each analyte between the reference standards and the urine samples. For all positive identifications, the variation in the ratios of the relative abundances of the monitored product ions was less than 20%, and the variation in the ratios of the relative retention times was less than 2%.

The limits of detection (LODs) varied between 10 pg mL-1 and 5 ng mL-1. The ionization efficiencies with electrospray ionization (ESI) were clearly dependent on the proton affinities (PA) of the analytes. The PAs of the steroids having 3-one-4-ene structure are relatively high and these steroids are efficiently ionized via proton transfer. LODs

were typically between 10 and 100 pg mL-1. The other steroids have lower PAs and they formed ammonium adduct ions with decreased ionization efficiency, with LODs typically between 0.1 and 5 ng mL-1. The glucuronides formed either protonated molecules or ammonium adducts with relatively good sensitivity (LODs between 10 and 250 pg mL-1). The relative standard deviation of analyte (5/25 ng mL-1) spiked in artificial urine samples were < 15%, which is acceptable for profiling purposes. The profiles were determined from ratios of the relative intensity of the peak area of the ion chromatogram of an analyte to that of an internal standard. The results are presented in the heat map of Figure 6.1.

Analysis of nontargeted steroid glucuronides. Nontargeted steroid glucuronides in late pregnancy urine samples from the pool sample from late pregnancy were first screened by UPLC-MS. The most abundant peaks appeared at m/z 482, 512, 514, 528, and 530, and these ions were selected as precursor ions to measure product ion spectra by UPLC-MS/MS. The product ion spectra showed abundant ions formed by the losses of ammonia and glucuronide moiety together with one or two water molecules (Table 6.A.2.), which were selected for SRM (Table 6.A.1.). The spectra also showed that the ions m/z 482, 512, 514, 528, and 530 are ammonium adducts of glucuronides. Over 70 peaks were detected (Figure 6.3), of which the concentrations of 25 compounds were clearly increased during the pregnancy (Figure 6.1).

The compound in the SRM chromatogram of ion m/z 482 at RT 4.75 min eluted just before E3-16α- and E3-17-Glu (RT 5.05 and RT 5.16 min, respectively) (Figure 6.2). The accurate mass of the compound was m/z 482.23926, corresponding to the elemental composition C24H36O9N (ammonium adduct) with error of -0.520 ppm. These results suggest that the compound is E3-3-Glu. Five peaks were observed in the SRM chromatograms of m/z 512, one in m/z 514, eight in m/z 528, and eleven in m/z 530. The accurate masses of these four sets of compounds correspond to the elemental compositions C27H46O8N, C27H48O8N, C27H46O9N, and C27H48O9N, respectively (Table 6.A.2). The MS/MS spectra of the ions show product ions formed by the loss of glucuronide moiety, together with ammonia and one or two water molecules or ions derived from the glucuronide moiety, i.e. [Glu+H]+ (m/z 177), [Glu+H-H2O]+ (m/z 159), and [Glu+H-H2O]+ (m/z 141) (Table 6.A.2). The HRMS and MS/MS spectra show that the compounds are ammonium adducts of glucuronides and the elemental compositions of the respective aglycones are C21H34O2 (MW=318 g mol-1), C21H36O2 (MW=320 g mol-1), C21H34O3 (MW=336 g mol-1), and C21H36O3 (MW=338 g mol-1), corresponding to the elemental compositions of C21 steroids.

In an attempt at more detailed identification, the glucuronides of C21 steroids were also analyzed by GC-MS after LC-fractionation, hydrolysis, and MOEX-TMS derivatization (see sect. 2.5). Two of the compounds detected in the GC-MS total ion chromatograms were identified, with the aid of reference compounds, as P and AP. The mass spectra of five of the compounds detected by GC-MS correlated relatively well with the library spectra of the MOEX-TMS derivatives of different isomers of hydroxylated pregnanolones; matching factors varied between 700 and 950. The retention indexes of these compounds were in the range of 2750-3050, corresponding to the retention index range for pregnanes (2622-3105) presented in the literature [29,42]. Unambiguous identification of the five compounds was not possible owing to the lack of reference standards. However, the data suggest that the glucuronides of C21 steroids are glucuronides of progesterone metabolites. This proposal is supported by the increase in the concentrations of these C21 steroid glucuronides during the pregnancy.

Profiles. The normalized profiles of the steroids and steroid glucuronides whose concentrations clearly changed during the pregnancy are presented in the heat map in Figure 6.1. The concentrations of 11 of the 27 targeted steroids and steroid glucuronides (Table 6.1) increased noticeably during the pregnancy. The maximum concentrations were typically about 10 to 400 fold during pregnancy (Table 6.A.3). The concentrations of the 16 other targeted steroids were unchanged, changes were minor, or the concentrations were close to or under the LODs. Many of these other analytes were androgens or corticoids, which are not classical pregnancy steroids. In addition, the concentrations of 25 mostly unidentified C21 steroid glucuronides clearly increased during the pregnancy (Figure 6.1). The profiles of the product ion chromatograms of the C21 steroid glucuronides were compared for three late pregnancy samples collected from three different pregnancies (Figure 6.3). The profiles are closely similar, and the same C21 steroid glucuronides were detected in all three samples, indicating preliminarily that the changes in concentrations of these steroid glucuronides during pregnancy are generic.

In general, the profiles show that the concentrations of the steroids and steroid glucuronides gradually increased during the pregnancy, rapidly decreased during the delivery, and five days after the delivery were at the same level as in the control sample. The concentrations of PROG, P-Glu, AP-Glu, 5α-THDOC-3-Glu, and 5α-THDOC-21-Glu, DHEA and most of the unknown C21 steroid glucuronides began to increase immediately at the start of the pregnancy, increased during the pregnancy, and sharply decreased after delivery. The profile of 17-OH-PREG and AP followed the same trend, but it was not detected at the beginning of the pregnancy, possibly owing to the limited sensitivity of the method for that compound. These results agree with previously reported ones, which showed increased concentrations of PROG, P, AP, and 5α-THDOC in blood during pregnancy [24,28,41].

The concentrations of E2-3-Glu and E2-17-Glu started to increase immediately at the beginning of the pregnancy, increased during the pregnancy, but decreased sharply about one week before the delivery. The concentrations of E3-3-Glu and E3-16/17-Glu behaved differently from that of E2-3-Glu and E2-17-Glu: their concentration started to increase immediately at the beginning of the pregnancy (see Table 6.A.3), but concentrations began to increase more rapidly between days 73 and 101, increased further thereafter, and decreased rapidly after delivery.

There are no earlier studies dealing with glucuronides of E2 and E3 during pregnancy, but the concentrations of E2 and E3 in blood and saliva have been shown to increase during pregnancy [42-44]. Moreover, the E3/E2 concentration ratio increases in late pregnancy blood, creating an estrogenic environment during the delivery [45]. Our results are consistent with these earlier findings, suggesting that the glucuronides of E2 and E3 could provide valuable biomarkers to follow developments in late pregnancy. This proposal now needs to be evaluated through study of a larger set of pregnant women.

The data show a clear decrease in the concentrations of steroids and steroid glucuronides in the samples collected on days 158 and 214 of pregnancy. The subject had painful overnight contractions on days 146, 149, 167, and 215, and the decreases in the concentrations are dated to the same period, suggesting a link between the concentrations of the steroids and a strong contractions.

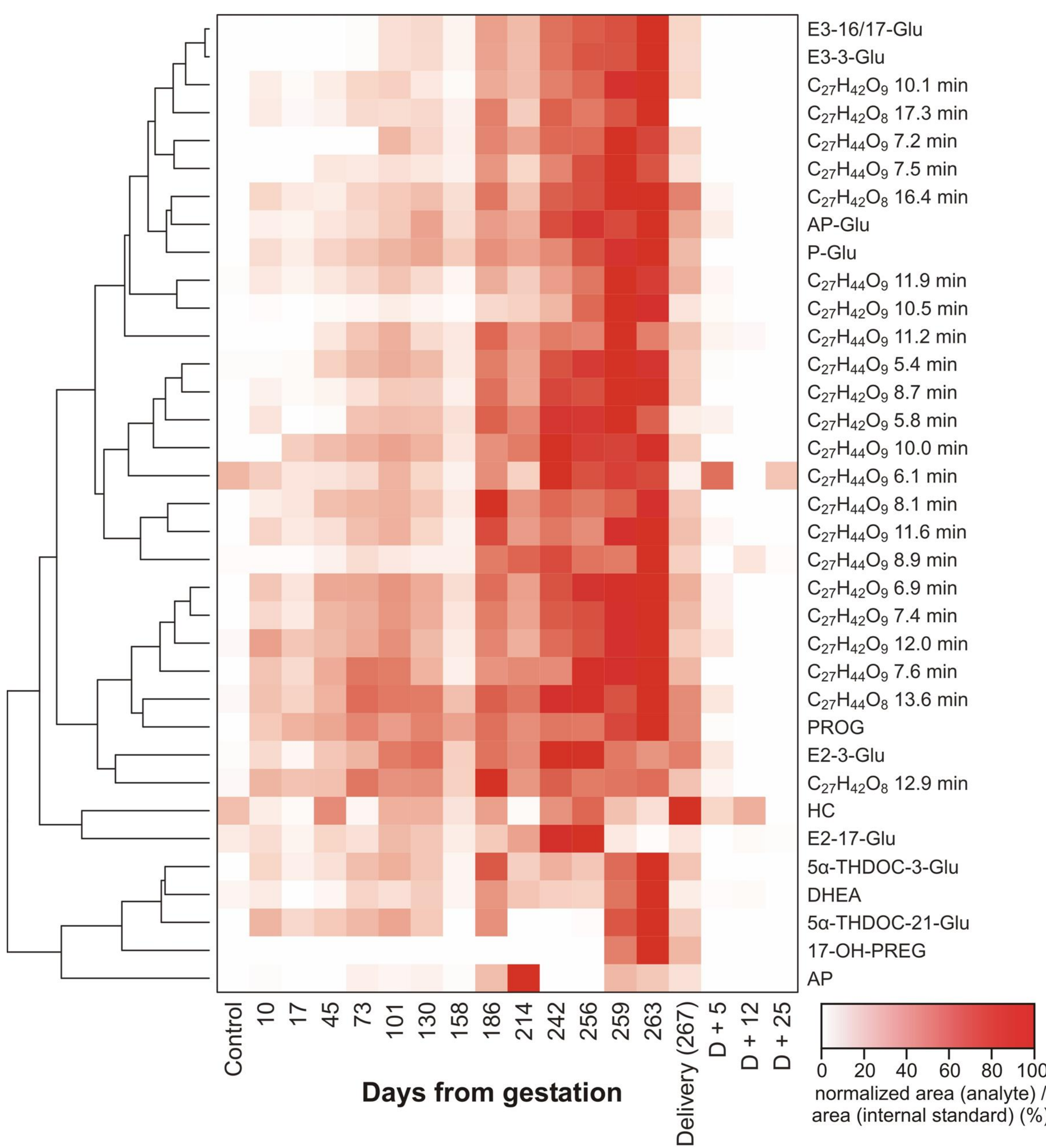


Figure 6.1. *Heat map of normalized responses of steroids and steroid glucuronides during pregnancy.*

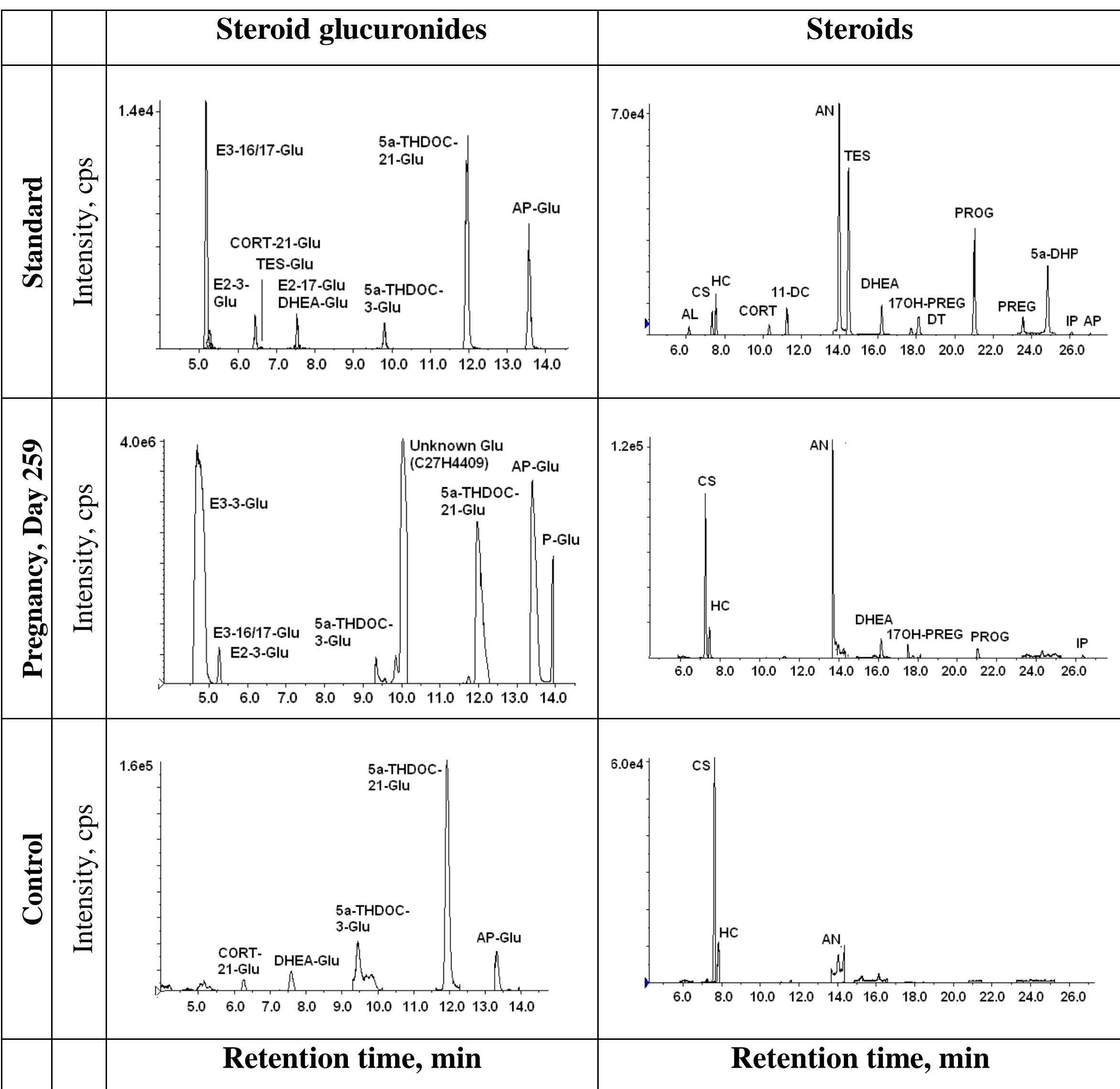


Figure 6.2. *SRM chromatograms of standard (analytes are spiked to synthetic urine, concentration 10 or 50 ng/ml), pregnancy urine collected 259 days after gestation, and non-pregnancy control urine measured by UPLC-MS/MS. Abbreviations of steroids and steroid glucuronides are presented in Table 6.1.*

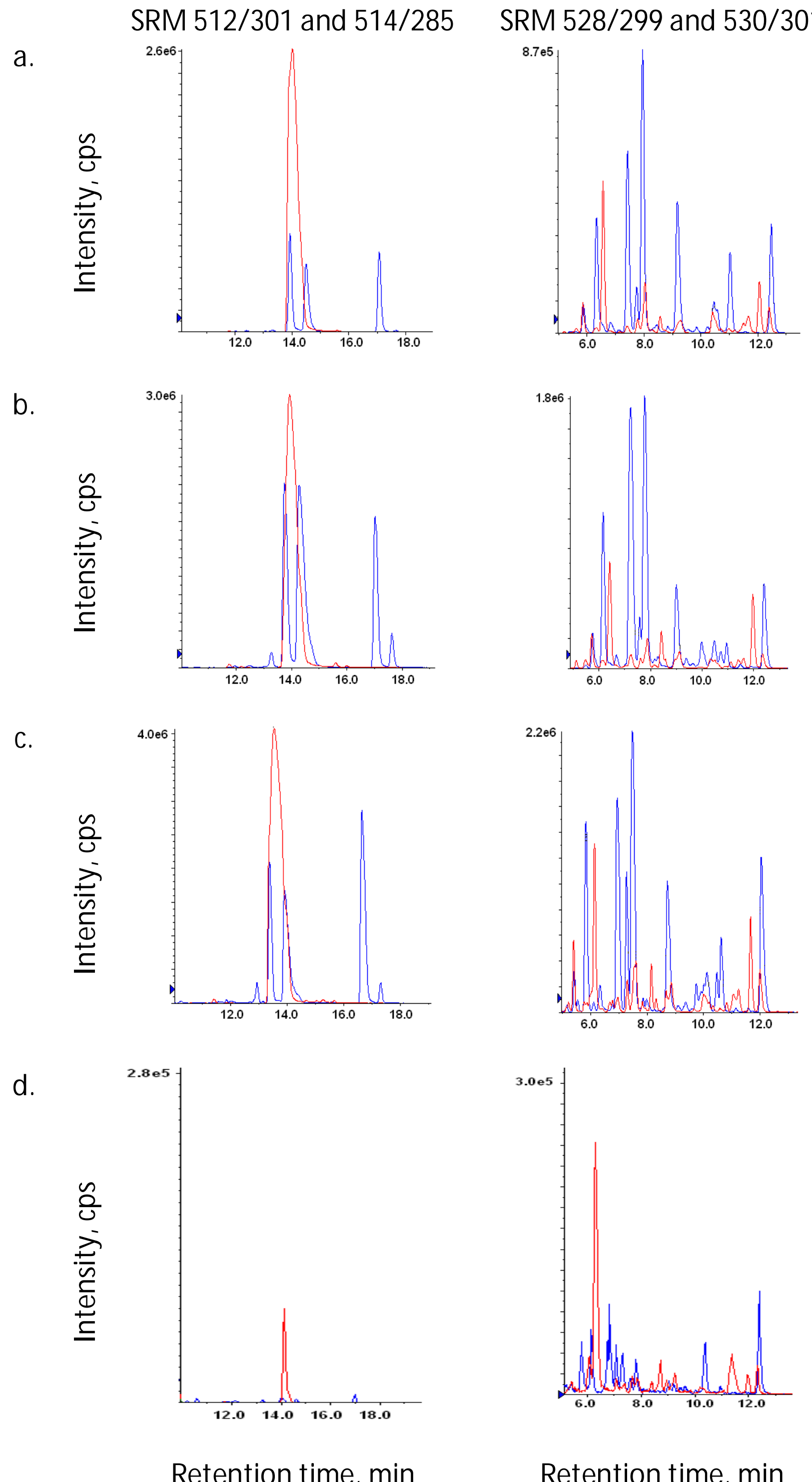


Figure 6.3. Comparison of SRM chromatograms of nontargeted steroid glucuronides in three pregnancies. a) Sample of volunteer 1 collected at week 37+1, b) Sample of volunteer 2 collected at week 36+4, c) Sample of volunteer 1 from an earlier pregnancy collected at week 35+0, and d) Control sample. SRM chromatogram of *m/z* 512/301 ($C_{27}H_{42}O_8$) and *m/z* 528/299 ($C_{27}H_{42}O_9$) are in blue and *m/z* 514/285 ($C_{27}H_{44}O_8$) and *m/z* 530/301 for ($C_{27}H_{44}O_9$) in red.

Table 6.A.2. *Identification and characterisation of C_{21}-glucuronides: Exact masses and MS/MS spectra. The urine sample analyzed was collected at day 259 of pregnancy. The declustering potentials and collision energies were same as presented in MS/MS measurements in Table 6.A.1.*

Elemental composition	*HRMS*	*MS/MS Spectra*										
RT, min	*Exact mass (error, ppm)* *for ammonium adduct of the steroid glucuronide*	$[Glu+H-2H_2O]^+$	$[Glu+H-H_2O]^+$	$[Glu+H]^+$	From ring structure	$[M+H-Glu-3H_2O]^+$	$[M+H-Glu-2H_2O]^+$	$[M+H-Glu-H_2O]^+$	$[M+H-Glu]^+$	$[M+H-H_2O]^+$	$[M+H]^+$	$[M+NH_4]^+$
$C_{27}H_{42}O_8$	Product ions of *m/z* 512	m/z 141	m/z 159	m/z 177	m/z 257	m/z 265	m/z 283	m/z 301	m/z 319	m/z 477	m/z 495	m/z 512
12.9	512.32264 (0.586)	10	5	4	7	ND	44	91	25	37	9	30
13.6	512.32264 (0.586)	24	8	14	3	ND	29	100	31	13	1	4
14.0	512.32234 (0.000)	22	9	14	3	ND	17	100	26	8	0	3
16.4	512.32234 (0.000)	2	3	0	2	ND	28	100	13	25	7	7
17.3	512.32222 (-0.234)	11	6	4	11	ND	33	84	100	11	4	53
$C_{27}H_{44}O_8$	Product ions of *m/z* 514	m/z 141	m/z 159	m/z 177	m/z 259	m/z 267	m/z 285	m/z 303	m/z 321	m/z 461	m/z 497	m/z 514
13.6	514.33790 (-0.162)	8	3	8	2	ND	100	4	2	10	0	11
$C_{27}H_{42}O_9$	Product ions of *m/z* 528	m/z 141	m/z 159	m/z 177	m/z 255, m/z 257	m/z 281	m/z 299	m/z 317	m/z 335	m/z 493	m/z 511	m/z 528
5.8	528.31740 (0.265)	2	4	1	1,2	19	53	100	2	4	3	2
6.9	528.31733 (0.132)	4	4	1	2,1	19	80	100	11	6	2	19
7.4	528.31733 (0.132)	2	4	2	2,0	14	100	55	10	7	2	2
8.7	528.31733 (0.132)	14	7	9	4,7	11	71	65	100	12	2	18
10.1	528.31703 (-0.435)	5	4	0	1,1	9	24	33	100	16	3	13
10.5	528.31727 (0.019)	4	3	0	4,4	18	39	19	100	6	3	11
12.0	528.31740 (0.265)	5	3	2	5,7	19	87	89	100	17	11	5
$C_{27}H_{44}O_9$	Product ions of *m/z* 530	m/z 141	m/z 159	m/z 177	m/z 257	m/z 283	m/z 301	m/z 319	m/z 337	m/z 495	m/z 513	m/z 530
5.4	530.33308 (0.321)	5	7	0	22	51	100	6	0	4	0	0
6.1	530.33278 (-0.245)	3	6	6	8	40	100	28	5	2	3	0
7.2	530.33308 (0.321)	6	8	6	15	60	100	18	0	8	2	13
7.5	530.33296 (0.094)	17	13	0	0	38	100	83	8	17	0	0
7.6	530.33278 (-0.245)	4	7	11	6	36	100	19	0	0	6	17
8.1	530.33339 (0.905)	9	9	7	24	47	100	42	7	7	9	0
8.9	530.33284 (-0.132)	7	10	5	10	47	27	100	5	34	0	3
10.0	530.33253 (-0.717)	37	5	18	68	74	79	58	100	21	5	32
11.2	530.33257 (-0.641)	13	16	5	33	42	100	42	13	15	13	0
11.6	530.33257 (-0.641)	13	16	4	33	42	100	42	13	15	13	0
11.9	530. 33278 (-0.245)	13	7	7	6	46	100	49	37	11	20	3

Table 6.A.3. *Fold changes of analyte responces: sample of maximum intensity during the pregnancy versus. day 10 sample and day 10 sample versus control (nonpregnancy) sample.*

Compound	**Fold**	
	Maximum/ day 10	**Day 10/ control**
E3-16/17-Glu	375	43.1
E3-3-Glu	384	43.1
$C_{27}H_{42}0_{9}$ 10.1min	20.3	NA (S/N 264)
$C_{27}H_{42}0_{8}$ 17.3 min	19.0	19.9
$C_{27}H_{44}0_{9}$ 7.2 min	NA (S/N 210)	NA (-)
$C_{27}H_{44}0_{9}$ 7.5 min	NA (S/N 318)	NA (-)
$C_{27}H_{42}0_{8}$ 16.4min	12.7	17.8
AP-Glu	28.3	12.2
P-Glu	10.8	NA (S/N 309)
$C_{27}H_{44}0_{9}$ 11.9 min	14.6	NA (S/N 27)
$C_{27}H_{42}0_{9}$ 10.5 min	57.2	12.0
$C_{27}H_{44}0_{9}$ 11.2 min	1.4	NA (S/N 51)
$C_{27}H_{44}0_{9}$ 5.4 min	45.2	NA (-)
$C_{27}H_{42}0_{9}$ 8.7 min	22.4	20.8
$C_{27}H_{42}0_{9}$ 5.8 min	13.8	20.0
$C_{27}H_{44}0_{9}$ 10.0 min	NA (S/N 85)	NA (-)
$C_{27}H_{44}0_{9}$ 6.1 min	6.0	-1.6
$C_{27}H_{44}0_{9}$ 8.1 min	20.5	NA (S/N 15)
$C_{27}H_{44}0_{9}$ 11.6 min	11.6	NA (S/N 116)
$C_{27}H_{44}0_{9}$ 8.9 min	2.5	NA (S/N 103)
$C_{27}H_{42}0_{9}$ 6.9 min	6.3	25.1
$C_{27}H_{42}0_{9}$ 7.4 min	9.5	28.0
$C_{27}H_{42}0_{9}$ 12.0 min	3.2	13.8
$C_{27}H_{44}0_{9}$ 7.6 min	6.7	9.3
$C_{27}H_{44}0_{8}$ 13.6 min	6.7	9.3
PROG	6.6	11.9
E2-3-Glu	10.9	7.0
$C_{27}H_{42}0_{8}$ 12.9 min	4.0	6.8
HC	11.8	-2.5
E2-17-Glu	13.1	1.7
5α-THDOC-3-Glu	6.9	36.4
DHEA	19.7	1.0
5α-THDOC-21-Glu	4.5	24.1
17OH-PREG	NA (S/N 13)	NA (-)
AP	120	7.9

NA: not available because the analyte was not detected in the control sample or in day 10 samples. In these cases S/N values are presented for the day 10 sample or for the sample of maximum intensity, (-) = not detected in day 10 sample.

6.4 Conclusions

A UPLC-ESI-MS/MS method was developed for the analysis of steroids and steroid glucuronides in urine. The method is fast and simple because the glucuronides can be analyzed in their intact form without time-consuming hydrolysis or derivatization steps. The sensitivity of UPLC-ESI-MS/MS is not a limiting factor since ESI provides high ionization efficiency, and the concentrations of steroid glucuronides are typically high in urine samples. The method was applied in determination of profiles of targeted and nontargeted steroids and steroid glucuronides during pregnancy. The concentrations of glucuronides were significantly higher than those of the respective aglycones, and their concentrations clearly changed during the pregnancy. Steroid glucuronides may therefore provide a valuable tool for following the progress of pregnancy. More detailed studies with a larger set of pregnant women are now needed to allow solid conclusions on the usefulness of steroid glucuronides as biomarkers during pregnancy.

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6.6 References

[1] R.H. Tukey, C.P. Strassburg, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 581-616.
[2] É. Lévesque, D. Turgeon, J.S. Carrier, V. Montminy, M. Baulieu, A. Bélanger, Biochem. 40(13) (2001) 3869-3881.
[3] C.P. Strassburg, M.P. Manns, R.H. Tukey, J. Biol. Chem. 273 (1998) 8719-8726.
[4] D.W. Hum, A. Bélanger, É. Lévesque, O. Barbier, M. Baulieu, C. Albert, M. Vallée, C. Guillemette, A. Tchernof, D. Turkeon, S. Dubois, J. Steroid Biochem. Molec. Biol. 69 (1999) 413-423.
[5] C.D. King, G.R. Rios, M.D. Green, T.R. Tephly, Curr. Drug Metab. 1(2) (2000) 143-161.
[6] M.B. Fisher, M.F. Paine, T.J. Strelevitz, S.A. Wrighton, Drug Metab. Rev. 33 (2001) 273-297.
[7] G.J. Pepe, E.D. Albrecht, Endocr. Rev. 16 (1995) 608-648.
[8] L. Milewich, C. Gomez-Sanchez, J.D. Madden, P.C. MacDonald, Gynecol. Obstet. Invest. 6 (1970) 291-306.
[9] C.R. Jr Parker, R.B. Everett, J.G. Jr Quirk, P.J. Whalley, N.F. Gant, Am. J. Obstet. Gynecol. 135 (1979) 778-782.
[10] B.E. Pearson Murphy, S.I. Steinberg, F.Y. Hu, C.M. Allison, J Clin. Endocrin. Metab. 86(12) (2001) 5981- 5987.
[11] K.F. Stoa, A. Bessesen, J. Steroid Biochem. 6 (1975) 21-25.

[12] A. Pařízek, M. Hill, R. Kancheva, H. Havlíková, L. Kancheva, J. Cindr, A. Pašková, V. Pouzar, I. Černý, P. Drbohlav, Z. Hájek, L. Stárka, J. Clin. Endocr. Metab. 90 (2005) 395-403.
[13] P.W. Nathaniels, Eur. J Obstet. Gynec. Reprod. Biol. 78 (1998) 127-132.
[14] W.X. Wu, X.H. Ma, T. Coksaygan, K. Chakrabarty, V. Collins, J. Rose, P.W. Nathanielsz, Endocrinology 145 (2004) 1444-1452.
[15] M. Hill, A. Parízek, R. Kancheva. M. Dunsková, M. Velíková, L. Kříž, M. Klímková, A. Pasková, Z. Žižka, P. Matucha, M. Meloun, L. Stárka. J. Steroid Biochem. Molec. Biol. 121 (2010) 594-610.
[16] M.D. Majewska N.L. Harrison, R.D Schwartz, J.L. Baker, S.M. Paul, Science 232 (1986) 1004-1007.
[17] M.D. Majewska, S. Demirgören, E.D. London, Eur J Pharmacol 189 (1990) 307-315.
[18] F.S. Wu, T.T. Gibbs, D.H. Farb, Mol Pharmacol. 40 (1991) 333-336.
[19] M. Hill, A. Parízek, D. Cibula, R.Kancheva, J.E. Jirásek, M. Jirkovská, M. Velíková, J. Kubátová, M. Klímková, A. Pašková, Z. Žižka, L. Kancheva, H. Kazihnitková, L. Zamrazilová, L., Stárka, J. Steroid Biochem. Mol. Biol. 122 (2010) 114-132.
[20] R.E. Nappi, F. Petraglia, S. Luisi, F. Polatti, C. Farina, A.R. Genazzani, Obstet Gynec. 97(1) (2001) 77-80.
[21] A.B. Brussaard, J. Wossink, J.C. Lodder, K.S. Kits, Proc. Natl. Acad. Sci. U.S.A. 97(7) (2000) 3625-3630.
[22] A.B. Brussard, K.S. Kits, R.E. Baker, W.P. Willems, J.W. Leyting-Vermeulen, P. Voorn, A.B. Smit, R.J. Bicknell, A.E. Herbison. Neuron 19(5) (1997) 1103-1114.
[23] S. Luisi, F. Petraglia, C. Benedetto, R.E. Nappi, F. Bernardi, M. Fadalti, M. Reis, M. Luisi, A.R. Genazzani, J. Clin. Endocr. Metab. 85 (7) (2008) 2429-2433.
[24] A.M. Paoletti, S. Romagnino, R. Contu, M.M. Orru, M.F. Marotto, P. Zedda, S. Lello, G. Biggio, A. Concas, G.B. Melis, Psychoneuroendocr. 31 (4) (2006) 485-492.
[25] J.W. Honour, Clin. Chem. Acta 313 (2001) 45-50.
[26] A.O. Chan, N.T. Taylor, S.C. Tiu, C.C. Shek, Steroids 73 (2008) 828-837.
[27] B.G. Wolthers, G.P. Kraan, J. Chrom. A. 843 (1999) 247-274.
[28] S.E. Gilbert Evans, L.E. Ross, E.M. Sellers, R.H. Purdy, M.K. Romach, Gynecol. Endocr. 21(5) (2005) 268-279.
[29] L-J. Meng, J. Sjövall, J. Chrom. B 688 (1997) 11-26.
[30] L-J. Meng, H. Reyes, J. Palma, I. Hernandez, J. Ribalta, J. Sjövall, J. Hepatol. 27 (1997) 346-357.
[31] M.M. Kushnir, A.L. Rockwood, W.L. Roberts, B. Yue, J. Bergquist, A.W. Meikle, Clin. Biochem. 44 (2011) 77-88.
[32] V.M. Carvalho, J. Chrom. B 883-884 (2012) 50-58.
[33] F. Biuarelli, F. Coccioli, M. Merolle, B. Neri, A. Terracciono, Anal. Chim. Acta 526(2) (2004) 113-120.
[34] P. Caron, E. Audet-Walsh, J. Lepine, A. Belanger, C. Guillemette, Anal. Chem. 81 (2009) 10143-10148.
[35] S. Ikegawa, M. Hasegawa, R. Okihara, C. Shimidzu, H. Chiba, T. Iida, K. Mitamura, Anal. Chem. 81(24) (2009) 10124-10135.
[36] S.E. Kallonen, A. Tammimäki, P. Piepponen, H. Raattamaa, R.A. Ketola, R.K. Kostiainen, Anal. Chim. Acta 651 (1) (2009) 69-74.

[37] S.E. Jäntti, A. Tammimäki, H. Raattamaa, P. Piepponen, R. Kostiainen, R.A. Ketola, Anal. Chem. 82(8) (2010) 3168-3175.
[38] R.A. Ketola, K.S. Hakala. Curr. Drug Metab. 11 (2010) 561-582.
[39] S.E. Jäntti, A. Kiriazis, R. Reinilä, R.K. Kostiainen, R.A. Ketola, Steroids 72(3) (2007) 287-296.
[40] H. Hedelin, L. Grenabo, S. Pettersson, J. Urol. 136 (1986) 743-745.
[41] M.J. Ebner, D.I. Corol, H. Havlíková, J.W. Honour and J.P. Fry, Endocrinol. 147(1) (2006) 179-190.
[42] O.P. Soldin, T. Guo, E. Weiderpass, R.E. Tractenberg, L. Hilakivi-Clarke, S.J. Soldin, Fertil. Steril. 84(3) (2005) 701-710.
[43] J. Darne, H.H McGarrigle, G.C. Lachelin, Br. J. Obstet. Gynaec. 94 (1987) 227-235.
[44] H.L. Hedriana, C.J. Mundro. E.M. Eby-Wilkens, B.I. Lasley, Amer. J. Obstet. Gynec. 184 (2) (2001) 123-130.
[45] R. Smith, J. I. Smith, X. Shen, P.J. Engel, M.E. Bowman, S.A. McGrath, A.M. Bisits, P. McElduff, W.B. Giles, D.W. Smith, J. Clin. Endocr. Metab. 94(6) (2009) 2066-2074.

7 Conclusions

The main objective of the study was to develop sample preparation and LC-MS/MS methods for the analysis of steroid and steroids conjugates from biological matrices with good selectivities and sensitivities. In the studies of steroids and their metabolites in tissues and biofluids, the first step is to identify relevant target compounds in the studied samples. However, to be able to do that, adequate analytical methods are needed, and often this requires the use of reference materials. Commercial sources of steroid glucuronide standards are limited or unavailable, and therefore, the synthesis of steroid glucuronides is needed to determine the presence and function of steroid glucuronides in organs of humans or animals.

7.1 Enzymatic synthesis

In our study, the reference materials were produced using enzymatic synthesis, and five stereochemically pure steroid glucuronide conjugates were recovered in milligram amounts with yields of 10–78%. Steroids, for which regionally isomeric mono-glucuronides can be produced, specificity of the glucuronidation site can, in some cases, be differentiated by selection of microsomal species. In our study, porcine and bovine livers were tested for eight steroid aclycones, because they are easily and inexpensively available, and painful and time consuming enzyme activity induction with Aroclor 1254 can be avoided (typically used with rat liver). Both bovine and porcine liver microsomes efficiently produced all the steroid glucuronides studied, whereas rat liver microsomes produced glucuronides efficiently only for three of the steroids. Since porcine liver apparently has a relatively large amount of dehydrogenase enzyme, its microsomes produced dehydrogenated steroids and their glucuronides as side products, as well as various regional isomers in which the site of glucuronidation varied. In contrast, bovine liver microsomes mainly produced a single major glucuronidation product and few dehydrogenation products and gave the best overall yield for two-third of the steroids tested, and good yields for the other steroids. Regional glucuronide isomers can be formed to all hydroxyl groups, but the glucuronidation of 11- and 17α-OH were found to be inefficient in the case where there is steric hindrance by the neighbouring rings and carbon side chains. For that reason the 21-glucuronide was detected as the main product in all the corticoids.

Before steroid glucuronides can be used as reference materials, the product has to be purified, for example by using LLE or SPE, and the structure has to be confirmed. In our study purification was done by an Oasis HLB cartridge for those synthesis products which yielded mainly one monoglucuronide as a product. For steroids with regional glucuronide monoisomers or glucuronides of dehydrogenation impurities, a more selective LC fractionation was required for purification. Reference materials were characterized by nuclear magnetic resonance spectroscopy (NMR), LC-MS/MS, and high resolution mass spectrometry to substantiate the correct structure of the product. Reliable identification with a high degree of purity (>85–90%) was obtained for all the steroid glucuronides

synthesised. In conclusion, enzymatic synthesis proved to be a fast and relative inexpensive way to produce milligram amounts of steroid glucuronides, especially if the reference material is not aimed at quantitative purposes and the small amounts of impurities can be tolerated. Adequate purification and characterisation of synthesis products are more time demanding and increase the costs of the synthesis, but this is still the fastest and most inexpensive way to produce small amounts of new steroid glucuronides. An advantage of enzymatic synthesis is that stereochemically pure products can be produced easily, which is not self-evident in the case of chemical synthesis. The synthesis procedure can be easily applied also to other types of steroids without long and time-consuming optimisation. The major disadvantage of enzymatic syntheses is that it is not suitable for large volume glucuronide synthesis.

7.2 Sample preparation

In the development of analytical methods for analysis and for the identification of the compounds, sample preparation plays an essential role. Brain tissue is a highly complex matrix containing a large variety of lipids, salts, proteins and peptides. Many of the lipids are abundantly present in the brain, and they can strongly suppress and interfere LC-MS, including blocking of the columns and shifts in retention times. We also observed the same problems at the beginning of the study, when we used sample purification method found in the literature [Liu et al., 2003], in which steroids were extracted from rat brain samples using ethanol, and the extracts were purified with several steps of SPE. To overcome the problems, we used an acetone-ethanol mixture instead of ethanol to decrease the co-extraction of the interfering lipids, and Oasis MCX mixed mode cation exchange cartridges to remove the rest of the lipids. The developed sample preparation procedure provided efficient extraction efficiency and sufficient purification for steroids and their conjugates from brain tissue samples. Urine samples are relative easy to process compared to brain samples. In comparison to brain samples urine contains high concentrations of salts and certain endogenous compounds which can easily be removed by using simple methods based on SPE or LLE. Our objective in the method development was to combine the analysis of steroid conjugates and steroids using the same method with good recovery, and thus, SPE was also chosen for the urine samples. The Oasis HLB cartridges were used in the purification of urine samples.

7.3 Analysis Methods

In RIA specific antibodies are needed for analysis, are not available for all steroid glucuronides. Cross reactions, selectivity and the use of radioactive labels restricts use of RIA.

In GC-MS conjugated steroids, glucuronides and sulphates, are traditionally hydrolysed prior to the analysis. Steroid conjugates are not sufficiently volatile or thermo stable compounds to be analysed with GC-MS in their intact form. The methods containing a hydrolysis step do not provide identification of the conjugates with absolute certainty, even if specific enzymes such as β-glucuronidase or sulphatase are utilized, and the origin of hydrolysis can remain unclear or incomplete hydrolysis can affect the results obtained. When enzymatic hydrolysis is used, formation of side products is also common problem, that can alter results.

GC-MS, LC-APPI-MS/MS and LC-APCI-MS/MS are effective methods for steroids hormones with low proton affinity and high lipofilicity. Although all these methods preclude the direct analysis of steroid hormone conjugates due to the dissociation of the conjugates in EI, APCI and APPI. ESI provides a very soft ionisation technique and therefore LC-ESI-MS/MS is the only method suitable for the simultaneous analysis of non-conjugated steroids and intact steroid glucuronides and sulphates.

Steroids with a 3-one-4-ene structure (many of corticoids and androgens) have relatively high proton affinity, and they are efficiently protonated using positive polarity and ESI. Steroids with hydroxyl groups or keto-functionalities without surrounding double-bond conjugation, as well as their steroid glucuronide conjugates, are not efficiently protonated due to their relatively low proton affinities. They can be ionized via ammonium adduct formation in LC-ESI/MS by adding low concentrations of volatile ammonium salts (e.g. formiate of acetate) to the eluent. However, ammonium adducts are quite easily fragmented in the ion source and for that reason optimisation of ESI parameters is substantial. Ion source temperature, nebuliser gas flow, curtain gas flow, and the geometry of ion source strongly affect the sensitivity obtained. We have recognized that those ion sources, which do not use thermal energy in desolvation process, provide best sensitivity for ammonium adducts of steroids.

Protonated molecule or ammonium adduct was chosen as the precursor ion for MS/MS analysis. Sodium adducts or other adducts were not observed in MS spectra. The steroids studied fragmented in MS/MS mostly following the same general pathway. In positive ion mode sequential losses of water molecules were typical for all steroids and characteristically the product ions formed by the losses of water molecules from hydroxyl groups were more intense than those from oxo groups. All the steroids studied produced typically numerous intensive and selective product ions formed by the fragmentation of the ring structure. The product ions m/z 97, 109 or 121 originating from the A-ring are characteristic for steroids with a 3-one-4-ene structure (many corticoids and androgens). Same results are obtained also by others. The structure characteristic product ions for steroids glucuronides were formed by the neutral loss of the glucuronide moiety followed by the loss of one or two water molecules. The MS/MS spectra of the steroid glucuronides also showed typical non-specific fragments formed by the loss of water molecule(s) and ions derived from the glucuronide fragment ion (e.g. m/z 177, 159 and 141).

Negative ion ESI provides high ionisation efficiency for the steroids or their conjugates having an acidic group such as estrogens, steroid glucuronides and sulphates. The steroid sulphates were not ionized efficiently enough in the positive ion mode and therefore the only option was the negative ion mode. In general, LODs of steroid sulphates are at ng/ mL level in positive ion mode (detected as ammonium adducts) and at pg/ mL level in negative ion mode. In negative ion MS/MS steroid sulphates produced two very abundant but non-characteristic product ions $[SO_3]^-$ (m/z 80) and $[HSO_4]^-$ (m/z 97). Although, steroid glucuronides were efficiently ionized in the negative ion mode, the use of the positive ion mode was justified for steroid glucuronides, because the steroid glucuronides in positive ion MS/MS produced intense structure specific product ions, but in negative ion mode non-characteristic product ions originated from the glucuronide fragment ion (m/z 175). In addition, the positive mode was the method of choice because some steroids and steroid glucuronides eluted in the same measurement section, and switching between the positive and negative modes was too slow with the instruments available for the study. Of course two separate runs can be made, one in the positive ion mode and the other in the negative ion mode, but this would result in non-desired and prolonged analysis times. On the contrary fast polarity switching allows analysis of positive and negative ions within same run in most modern MS instruments.

To obtain maximum sensitivity and selectivity specific and intense product ions were chosen for selected reaction monitoring (SRM). Sensitivity and specificity of the LC-ESI/MS/MS method could be enhanced by careful selection of mobile phases, gradient and the temperature of the column. After optimisation, the methods were validated. The linear range of 3-4 magnitude was obtained ($R^2 > 0.996$) with good precision (RSD <15%). Except for E3-16-Glu and E3-17-Glu, all analytes, including α/β and positional isomers, were well separated by our UPLC and Capillary LC method. Detection limits of 6-100 pmol/l were obtained for steroid glucuronides, 10-30 pmol/l for steroid sulphates and 0.03-22 nmol/l for non-conjugated steroids, respectively. The results showed good quantitative performance for the methods.

7.3.1 Analysis of brain extracts

Using the developed CapLC-ESI-MS/MS method neurosteroid glucuronides were found for the first time in mouse brain samples extracted from hippocampus, hypothalamus, cortex and mid brain. The glucuronides were identified by comparing retention times and the relative abundances of two SRM transitions of each neurosteroid glucuronide between the reference and authentic samples, thus providing reliable identification. Six steroid glucuronides (E2-17-Glu, HC-21-Glu, TES-Glu, CORT-21-Glu, PREG-Glu, IP-Glu) and four non-conjugated steroids (HC, CORT, DHEA, and DHP) were measured within concentration range of 0.2-58 ng/g in mouse hippocampus and hypothalamus. The detection of neurosteroid glucuronides in rat brain raised the question of whether the glucuronides are biosynthetised *in situ* in the rodent brain. To get an answer to this question selected steroids (CORT, PREG and DHEA) were incubated *in vitro* using mouse and rat brain subcellular S9 fraction containing both cytosomal and microsomal enzymes. The results showed that the required enzymes exist in rodent brain, and glucuronidation of selected steroids was possible, and furthermore, that biosynthesis of neurosteroid glucuronides could be possible in the rodent brain.

Although the role of steroid glucuronidation in the brain remains unknown, several questions emerge: Is glucuronidation an elimination pathway for steroids in the brain? Do efflux transporters exist for steroid glucuronides? Are glucuronides back-hydrolysed to native steroids or do they accumulate in the brain? Have they neurotoxic, neuroprotective or pharmacological properties? Are steroid glucuronides present also in the human brain? Answering these questions requires thorough pharmacological experiments and even more sensitive analytical methods because many of the steroids exist at very low concentrations in brain samples. Although it was demonstrated in this work that neurosteroid glucuronides can be formed in brain, the possibility that the glucuronides are synthetized at peripheral tissues and transferred through blood brain barrier to brain cannot be excluded. This question could be studied by introducing stable isotope labelled steroids to the brain and to blood circulation and analysing the brain, CSF and plasma samples in LC-MS/MS.

7.3.2 Analysis of urine samples during pregnancy

The developed UPLC-ESI-MS/MS was used to analyse urine samples collected during the pregnancy of a health volunteer. Estrogens (E2, E3) and PROG are primary pregnancy steroids, which can be further metabolized via phase I and II reactions before secretion to the urine. High concentrations of estrogen glucuronides were detected in the urine samples. PROG was metabolised to a large spectrum of pregnane metabolites, detected mainly in urine as pregnane glucuronides. Over seventy steroid glucuronides, mainly originated from pregnanes, were detected in pregnancy urine.

In general the concentrations of the steroids or steroid glucuronides involved in pregnancy were altered during the pregnancy. The concentrations typically started to elevate immediately at the beginning of the pregnancy, they increased during the pregnancy, and sharply decreased during delivery. The concentrations of E2-3-Glu and E2-17-Glu started to increase immediately at the beginning of the pregnancy, increased during the pregnancy, but decreased sharply approximately one week before the delivery. The patterns of E3-3-Glu and E3-16/17-Glu concentrations were different from that of E2-3-Glu and E2-17-Glu: their concentration started to increase immediately at the beginning of the pregnancy, but increased more rapidly between days 73 and 101, increased further thereafter, and decreased rapidly during delivery.

Although there are no earlier studies dealing with glucuronides of E2 and E3 during pregnancy, it is known that the concentrations of E2 and E3 have been shown to increase in blood and saliva during pregnancy and the E3/E2 concentration ratio increases in late pregnancy blood, creating an estrogenic environment during the delivery. Our results are consistent with these earlier findings, suggesting that the glucuronides of E2 and E3 could provide valuable biomarkers to follow developments in late pregnancy. This proposal now needs to be evaluated in the study with a larger set of pregnant women.

The data show a clear decrease in the concentrations of steroids and steroid glucuronides in the samples collected on days 158 and 214 of pregnancy. The subject had painful overnight contractions on days 146, 149, 167, and 215, and the decreases in the concentrations are dated to the same period, suggesting a link between the concentrations of the steroids and a strong contractions.

As the concentrations of many steroids and steroid glucuronides increased and especially hydroxylated pregnane glucuronides were detected at high concentrations in the study, it can be suggested that they may also have neuroactive properties, if they can penetrate the blood brain barrier (BBB). If they have neuroactive properties, how do they affect mood, behaviour and stress during pregnancy? In order to get more reliable conclusions on the role of steroids and their glucuronides during the pregnancy, more detailed studies with larger number of subjects must be carried out. Also more work must be carried out to characterize the structures of all the detected steroid glucuronides. Also, non-targeted steroid profiling using high resolution mass spectrometry could be useful to study the role of steroids during the pregnancy.

8 References

Akwa Y. and Baulieu E.E., Dehydroepiandrosterone sulfate and dehydroepiandrosterone: Neuroactive Steroids. Curr. Opin. Endocri. Diabetes 7 (2000) 160-167.

Albrecht E.D. and Pepe G.J. Placental Steroid Hormone Biosynthesis in Primate Pregnancy. Endocr. Rev. 11 (1990) 124-150.

Ălvarez Sánchez B., Priego Capote F., Ruiz Jiménez J. and Lugue de Castro M.D. Automated solid-phase extraction for concentration and clean-up of female steroid hormones prior to liquid chromatography ionization-tandem mass spectrometry: An approach to lipidomics. J. Chrom. A 1207 (2008) 46-54.

Andrew R. Clinical measurement of steroid metabolism. Best Pract. Res. Clin. Endocr. Metab. 15 (2001) 1-16.

Antignac J-P., Brosseaud A., Gaudin-Hirret I., André F. and Le Bizec B. Analytic strategies for the direct mass spectrometric analysis of steroid and corticosteroids phase II metabolites. Steroids 70 (2005) 205-216.

Basu N.K., Kubota S., Meselhy M.R., Ciotti M., Chowdhury B., Hartori M. and Owens IS. Gastrointestinally distributed UDP-glucuronosyltransferase 1A10, which metabolizes estrogens and nonsteroidal anti-inflammatory drugs, depends upon phosphorylation. J. Biol. Chem. 279 (2004) 28320-28329.

Baulieu E.E. and Robel P. Neurosteroids: a new brain function? J. Steroid Biochem. Mol. Biol. 37 (1990) 395-403.

Baulieu E.E. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. Recent Prog. Horm. Res. 52 (1997) 1-32.

Baulieu E.E. Neurosteroids: A New Regulatory Function in the Nervous System. Humana Press Inc. Totowa, NJ. (1999) 1-25.

Baulieu M., Lévesque É., Hum D.W., and Bélanger, A. Isolation and characterization of a novel cDNA encoding a human UDP-glucuronosyltransferase active on C_{19} steroids. J. Biol. Chem. 271 (1996) 22855-22862.

Bélanger A., Hum D.W., Beaulieu M., Lévesque É., Guillemette C., Tchernof A., Bélanger G., Turgeon D., and Dubois, S. Characterization and Regulation of UDP-Glucuronosyltransferases in Steroid Target Tissues. J. Steroid Biochem. Molec. Biol. 65 (1998) 301-310.

Bélanger G., Barbier O., Hum D.W. and Bélanger A. Molecular cloning, expression and characterization of a monkey steroid UDP-glucuronosyltransferases, UGT2B19, that conjugates testosterone. Eur. J. Biochem. 260 (1999) 701-708.

Bélanger C., Hould F.S., Lebel S., Biron S., Brochu G. and Tchernof A. Omental and subcutaneous adipose tissue steroid levels in obese men. Steroids 71 (2006) 674-682.

Belelli, D. and Lambert, J. L. Neurosteroids: endogenous regulators of the GABA(A) receptor. Nat. Rev. Neurosci. 6 (2005) 565-575.

Best R. and Walker B.R. Additional value of measurement of urinary cortisone and unconjugated cortisol metabolites in assessing the activity of 11beta-hydroxysteroid dehydrogenase in vivo. Clin. Endocr. 47 (1997) 231-236.

Biuarelli F., Coccioli F., Merolle M., Neri B. and Terracciano A. Development of a liquid chromatography-tandem mass spectrometry method for the identification of natural androgen steroids and their conjugates in urine samples. Anal. Chim. Acta 526 (2004) 113-120.

Blonder J., Johann D.J., Veenstra T.D., Xiao Z., Emmert-Buck M.R., Ziegler R.G., Rodriguez-Canales J., Hanson J.A. and Xu X. Quantitation of Steroid Hormones in Thin Fresh Frozen Tissue Sections. Anal. Chem. 80 (2008) 8845-8852.

Bowalgaha K., Elliot D. J., Mackenzie P. I., Knights K. M. and Miners J. O. The glucuronidation of Δ4-3-Keto C19- and C21-hydroxysteroids by human liver microsomal and recombinant UDP-glucuronosyltransferases (UGTs): 6α- and 21-hydroxyprogesterone are selective substrates for UGT2B7. Drug Met. Dis. 35 (2007) 363-370.

Bowers L.D. and Sanaullah J. Direct measurement of steroid sulfate and glucuronide conjugates with high-performance liquid chromatography-mass spectrometry. J. Chromatogram. B 687 (1996) 61-68.

Brussard A.B., Kits K.S., Baker R.E., Willems W.P., Leyting-Vermeulen J.W., Voorn P., Smit A.B., Bicknell R.J. and Herbison A.E. Plasticity in fast synapticminhibition of adult oxytocin neurons caused by switch in $GABA_A$ receptor subunit expression. Neuron 19 (1997) 1103-1114

Brussaard A.B., Devay P., Leyting-Vermeulen J.L. and Kits K.S. Changes in properties and neurosteroid regulation of GABAergic synapses in the supraoptic nucleus during the mammalian female reproductive cycle. J. Physiol. 516 (1999) 513-524.

Brussaard A.B., Wossink J., Lodder J.C. and Kits K.S. Progesterone-metabolite prevents protein kinase C-dependent modulation of λ-aminobutyric acid type A receptors in oxytocin neurons. Proc. Acad. Sci. U.S.A. 97 (2000) 3625-3630.

Burchell B., Nebert D.W., Nelson D.R., Bock K.W., Iyanagi T., Jansen P.L.M., Lancet D., Mulder G.J., Chowdhury J.R., Siest G., Tephly T.R., and Mackenzie P.I. The UDP glucuronosyltransferase gene superfamily: Suggested nomenclature based on evolutionary divergence. DNA Cell Biol. 10 (1991) 487-494.

Caron P., Audet-Walsh E., Lepine J., Belanger A. and Guillemette C. Profiling Endogenous Serum Estrogen and Estrogen-Glucuronides by Liquid Chromatography-Tandem Mass Spectrometry. Anal. Chem. 81 (2009) 10143-10148.

Caruso D., Scurati S., Maschi O., De Angelis L., Roglio I., Giatti S., Garcia-Segura L.M. and Melcangi R.C. Evaluation of neuroactive steroid levels by liquid chromatography-tandem mass spectrometry in central and peripheral nervous system: Effect of diabetes. Neurochem. Int. 52 (2008) 560-568.

Ceglarek U., Kortz L., Leichtle A., Fiedler G.M., Kratzsch J. and Thiery J. Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography-triple quadrupole linear ion trap mass spectrometry. Clin. Chim. Acta 401 (2009) 114-118.

Chan A.O., Taylor N.T., Tiu S.C. and Shek C.C. Reference intervals of urinary steroid metabolites using gas chromatography-mass spectrometry in Chinese adults. Steroids 73 (2008) 828-837.

Cheng Z., Radominska-Pandya A., and Tephly T. Studies on the substrate specificity of human intestinal UDP-glucuronosyltransferases 1A8 and 1A10. Drug Met. Dis. 27 (1999) 1165-1170.

Choi M.H., Kim J.N. and Chung B.C. Rapid HPLC electrospray - tandem mass spectrometric assay for urinary testosterone and dihydrotestosterone glucuronides from patience with beging prostate hyperplasia. Clin. Chem. 49 (2003) 322-325.

Coffman B.L., King C.D., Rios G.R., and Tephly T.R. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y(268) and UGT2B7H(268). Drug Met. Dis. 26 (1998) 73-77.

Comer K.A., Falany J.L. and Falany C.N. Cloning and expression of human liver dehydroepiandrosterone sulphotransferase. Biochem J. 289 (1) (1993) 233-240.

Compagnone N.A. and Mellon S.H. Neurosteroids: Biosynthesis and function of these novel neuromodulators. Front.Neuroendocrin. 21 (2000) 1-56.

Corpéchot C., Robel P., Axelson M., Sjövall J. and Baulieu E.E. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc Natl. Acad. Sci. USA 78 (1981) 4704-4707.

Corpéchot C., Synguelakis M., Talha S., Axelson M, Sjövall J., Vihko R., Baulieu E.E. and Robel P. Pregnanolone and its sulfate ester in rat brain. Brain Res. 270 (1983) 119-125.

Corpéchot C., Young J., Calvel M., Wehrey C., Veltz J.N., Touyer G., Mouren M., Prasad V.V.K., Banner C., Sjövall J., Baulieu E.E. and Robel, P. Neurosteroids: 3α-hydroxy-5βpregnan-20-one and its precursors in the brain, plasma, and steroidogenic glands of male and female rats Endocr. 133 (1993) 1003-1009.

Dalvie D.E, and Loi C-M. Role and clinical consequences of human UDP-Glucuronosyltransferases Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition. 2012 John Wiley & Sons.

Dehennin L., Lafarge P., Dailly Ph., Bailloux D. and Lafarge J.-P. Combined profile of androgen glucuro- and sulfoconjugates in postcompetition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry. J. Chromatogr. B 687 (1996) 85-91.

Do Rego J.L., Tremblay Y., Luu-The V., Repetto E., Castel H., Vallarino M., Bélanger A., Pelletier G. and Vaudry H. Immunohistochemical localization and biological activity of the steroidogenic enzyme cytochrome P450 17a-hydroxylase/C17, 20-lyase (P450C17) in the frog brain and pituitary. J. Neurochem. 100 (2007) 251-268.

Do Rego J., Seong J., Burel D., Leprince J., Luu-The V., Tsutsui K., Tonon C.-M., Pelletier G. and Vaudry H. Neurosteroid Biosynthesis: Enzymatic pathways and neuroendocrine regulation ny neurotransmitters and neuropeptides. Front. Neuroendocr. 30 (2009) 259-301.

Draisci R., Palleschi L., Ferretti E., Lucentini L. and Cammarata, P. Quantitation of anabolic hormones and their metabolites in bovine serum and urine by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 870 (2000) 511-522.

Evans S.G., Ross L., Sellers E.M., Purdy R.H., Romach M. K. 3α-reduced neuroactive steroids and their precursors during pregnancy and the postpartum period. Gynecol. Endocr. 21(5) (2005) 268-279.

Ebner M.J., Corol D.I., Havlíková H., Honour J.W. and Fry J.P. Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain J.Endocr. 147 (2006) 179-190.

Falany C.N., Xie X., Wang J., Ferrer J. and Falany J.L. Molecular cloning and expression of novel sulphotransferase-like cDNAs from human and rat brain. Biochem. J. 346 (3) (2000) 857-864.

Finel M., Gardner-Stephen D., Bratton S., Mackenzie P.I. and Radominska-Pandya, A. Human UDP-Glucuronosyltransferase 1A5: Identification, Expression, and Activity. J. Pharmacol. Exp. Ther. 315(3) (2005) 1143-1149.

Fuda H., Lee Y.C., Shimizu C., Javitt N.B. and Strott C.A. Mutational analysis of human hydroxysteoid sulfotransferases SULT2B1 isoforms reveals that exon 1B of the SULT2B1 gene products cholesterol sulfotransferase, whereas exon 1A yields pregnenolone sulfotransferase J. Biol.Chem. 277 (2002) 36161-36166.

Furuta T. Namekawa T., Shibasaki H. and Kasuya Y. Simultaneous determination of tetrahydrocortisol and tetrahydrocortisone in human plasma and urine by stable isotope dilution mass spectrometry. J. Chrom. B 706 (2) (1998) 181-190.

Gall W.E., Zawada G., Mojarrabi B., Tephly T.R., Green M.D., Coffman B.L., Mackenzie P.I. and Radominska-Pandya A. Differential glucuronidation of bile acids, androgens and estrogens by human UGT1A3 and 2B7. J. Steroid Biochem. Molec. Biol. 70 (1999) 101-108.

Gamage N., Barnett A., Hempel N., Duggleby R.G., Windmil K.F., Martin J.L,. and McManus M.E. Human sulfotransferases and their role in chemical metabolism. Toxicol. Sci. 90(1) (2006) 5-22.

Gasior M., Carter, R.B. and Witkin, J. Neuroactive Steroids: Potential Therapeutic Use in neurological and Ppsychiatric Disorders. Trends Pharmacol. Sci. 20 (1999) 107-112.

Girard C., Barbier O., Turgeon D., and Bélanger A. Isolation and characterization of the monkey UGT2B30 gene that encodes a uridine diphosphate-glucuronosyltransferase enzyme active on mineralocorticoid, glucocorticoid, androgen and oestrogen hormones. Biochem. J. 365 (2002) 213-222.

Girard C., Barbier O., Veilleux G., El-Alfy M. and Bélanger A. Human uridine diphosphate-glucuronosyltransferase UGT2B7 conjugates mineralocorticoid and glucocorticoid metabolites. Endocr. 144(6) (2003) 2659-2668.

Green M.D., Oturu E.M., and Tephly, T.R. Stable expression of a human liver UDP-glucuronosyltransferase (UGT2B15) with activity toward steroid and xenobiotic substrates. Drug Met. Dis. 22 (1994) 799-805.

Green M.D. and Tephly T.R. Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. Drug Met. Dis. 24 (1996) 356-363.

Green M.D., King C.D., Mojarrabi B., Mackenzie P.I., and Tephly T.R. Glucuronidation of amines and other xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase 1A3. Drug Met. Dis. 26 (1998) 507-512.

Griffiths W., Liu S., Avelius G., Sjöval J. Derivatisation for the characterisation of neutral oxosteroids by electrospray and matrix-assisted laser desorption/ionisation tandem mass spectrometry: The girard P derivative. Rapid Comm. Mass Spectr. 17 (2003) 924-935.

Griffiths W., Alvelius G., Liu S., Sjöval J. High-energy collision-induced dissociation of oxosteroids derivatized to Girard hydrazones. Eur. J. Mass. Spectrom. 10 (2004) 63-88.

Guo T., Taylor R.L., Ravinder J.S., Singh J.R., Soldin S.J. Simultanoeus determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin. Chem. Acta 372 (2006) 76-82.

Guo T., Gu J., Soldin O.P., Singh J.R. and Soldin S.J. Rapid measurement of estrogens and their metabolites in human serum by liquid chromatography-tandem mass spectrometry without derivatization. Clin. Biochem. 41 (2008) 736-741.

Ha Y.W., Moon J-Y., Jung H-J., Chung B.C. and Choi M.H. Evaluation of plasma enzyme activities using gas chromatography-mass spectrometry based steroid signatures. J. Chrom. B 877 (2009) 4125-4132.

Hauser B., Descner T. and Boesch C. Development of a liquid chromatography-tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine. J. Chrom. B 862 (2008) 100-112.

Higashi T., Takido N. and Shimada K. Studies on neurosteroids XVII. Analysis of stress-induced changes in neurosteroid levels in rat brains using liquid chromatography-electron capture atmospheric pressure chemical ionization-mass spectrometry. Steroids 70(1) (2005) 1-11.

Higashi T., Ninomiya Y., Iwaki N., Yamauchi A., Takayma N., Shimada K. Studies on neurosteroids XVIII: LC-MS analysis of changes in rat brain and serum testosterone levels induced by immobilization stress and ethanol administration. Steroids 71 (2006) 609-617.

Higashi T., Nagahama A., Otomi N., Shimada K. Studies on neurosteroids XIX: Development and validation of liquid chromatography-tandem mass spectrometric method for determination of 5α-reduced pregnane-type neurosteroids in rat brain and serum, J. Chrom. B 848 (2007) 188-199.

Higashi T., Yokoi H., Nagura Y., Nishio T. and Shimada K. Studies on neurosteroids XXIV. Determination of neuroactive androgens, androsterone and 5α-androstane-3α,17β-diol, in rat brain and serum using liquid chromatography-tandem mass spectrometry. Biomed. Chromatogr. 22 (2008) 1434-1441.

Hill M., Parízek A., Cibula D., Kancheva R., Jirásek J.E., Jirkovská M., Velíková M., Kubátová J., Klímková M., Pašková A., Žižka Z., Kancheva L., Kazihnitková H., Zamrazilová L., Stárka L. Steroid metabolome in fetal and maternal body fluids in human late pregnancy. J. Steroid Biochem. & Mol. Biol. 122 (2010) 114-132.

Hill M., Parízek A., Kancheva R., Dunsková M., Velíková M., Kříž L., Klímková M., Pasková A., Žižka Z., Matucha P., Meloun M., Stárka L.. Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and inamniotic fluid in normal and preterm labor. J. Steroid Biochem. Molec. Biol. 121 (2010) 594-610.

Hum D.W., Bélanger A., Lévesque É., Barbier O., Beaulieu M., Albert C., Vallée M., Guillemette C., Tchernof A., Turgeon D., and Dubois S. Characterization of UDP-glucuronosyltransferases active on steroid hormones. J. Steroid Biochem. Molec. Biol. 69 (1999) 413-423.

Ikegawa S., Hasegawa M., Okihara R., Shimidzu C., Chiba H., Iida T. and Mitamura K. Simultaneous determination of twelve tetrahydrocorticosteroid glucuronides in human urine by liquid chromatography/electrospray ionization-linear ion trap mass spectrometry. Anal. Chem. 81 (24) (2009) 10124-10135.

Itäaho K., Mackenzie P.I., Ikushiro S-I. Miners J.O., and Finel M. The configuration of the 17-hydroxy group variably influences the glucuronidation of β-estradiol and epiestradiol by human UDP-glucuronosyltransferases. Drug Met. Dis. 36 (2008) 2307-2315.

Iwata T., Hirose T. and Yamaguchi M. Direct determination of estriol 3- and 16-glucuronides in pregnancy urine by column-switching high-performance liquid chromatography with fluorescence detection. J. Chrom. B 695 (1997) 201-207.

Jain M.R., Burade V.S. and Subhedar N. Neurosteroids: Novel Regulators of Neuronal Functions. Indian J. Pharmacol. 27 (1995) 214-221.

Jia Q., Hong M-F., Pan Z-X. and Orndorff S. Quantification of urine ketosteroid sulfates and glucuronides by high-performance liquid chromatography-ion trap mass spectrometry. J. Chrom. B 750 (2001) 81-91.

Jin C.J., Miners J.O., Lillywhite K.J., and Mackenzie P. cDNA cloning and expression of two new members of the human liver UDP-glucuronosyltranseferase 2B subfamily. Biochem. Biophys. Res. Comm. 194 (1993) 496-503.

Jin C.J., Mackenzie C. P. I. and Miners, J. O. The regio- and stereo-selectivity of C19 and C21 hydroxysteroid glucuronidation by UGT2B7 and UGT2B11. Arch. Biochem. Biophys. 341 (1997) 207-211.

Kancheva R., Hill M., Novák Z., Chrastina J., Velíková M., Kancheva L., Ríha I. and Stárka L. Peripheral neuroactive steroids may be as good as the steroids in the cerebrospinal fluid for the diagnostics of CNS disturbances. J. Steroid Biochem. Mol. Biol. 119 (2010) 35-44.

Kicman T.A. Biochemical and Physiological Aspects of Endogenous Androgens. Doping in Sports: Biochemical Principles, Effects and Analysis, Hand book of experimental Pharmacology 195 (2010) 25-64.

Kim Y-S., Zhang H. and Kim H-Y. Profiling neurosteroids in cerebrospinal fluids and plasma by gas chromatography/electron capture negative chemical ionization mass spectrometry. Anal. Biochem. 277 (2000) 187-195.

King C.D., Green M.D., Rios G.R., Coffman B.L., Owens I.S., Bishop W.P. and Tephly T.R. The glucuronidation of exogenous and endogenous compounds by stably expressed rat and human UDP-glucuronosyltransferase 1.1. Arch. Biochem. Biophys. 332 (1996) 92-100.

King C.D., Rios G.R., Assouline J.A. and Tephly T.R. Expression of UDP-glucuronosyltransferases (UGTs) 2B7 and 1A6 in thehuman brain and identification of 5-hydroxytryptamine as a substrate. Arch. Biochem. Biophys. 365 (1999) 156-62.

Koal T., Schmiederer D., Pham-Tuan H., Roehring C. and Rauh M. Standardized LC-MS/MS based steroid hormone profile-analysis. J. Steroid Biochem. Mol. Biol. 129 (2012) 129-138.

Kostiainen, J., Kauppila, T. Effect of eluent on the ionization process in liquid chromatography-mass spectrometry J. Chrom. A 1216 (2009) 685-699.

Lépine L., Bernard O., Plante M., Tétu B., Pelletier G., Labrie F., Bélanger A. and Guillemette C. Specificity and regioselectivity of the conjugation of estradiol, estrone, and their catecholestrogen and methoxyestrogen metabolites by human uridine diphospho-glucuronosyltransferases expressed in endometrium. J. Clin. Endocr. Metab. 89 (2004) 5222-5232.

Lévesque É., Turgeon D., Carrier J.S., Montminy V., Beaulieu M., and Bélanger A. Isolation and characterization of the UGT2B28 cDNA encoding a novel human steroid conjugating UDP-glucuronosyltransferase. Biochem. 40 (2001) 3869-3881.

Liere P., Akwa Y., Weill-Engerer S., Eychenne B., Pianos A., Robel P., Sjövall J., Schumacher M. and Baulieu E.E. Validation of an Analytical procedures to measure trace amounts of neurosteroids in brain tissue by gas chromatography - mass spectrometry. J. Chromatogr. B 739 (2000) 301-312.

Liere P., Pianos A., Eyechenne B., Cambourg A., Liu S., Griffiths W., Schumacher M., Sjovall J. and Baulieu E.E. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulphated counterparts in rodent brain. J. Lipid Res. 45 (2004) 2287-2302.

Liere P., Pianos A., Eyechenne B., Cambourg A., Bodin K., Griffiths W., Schumacher M., Baulieu E.E. and Sjövall J. Analysis of pregnenolone and dehydroepiandrosterone in rodent brain: cholesterol autoxidation is the key. J. Lipid Res. 50 (2009) 2430-2444.

Lindsay J., Wang, L-L, Li Y. , Zhou S.-F. Structure, function and polymorphism of human cytosolic sulfotransferases. Curr. Drug Metab. 9 (2008) 99-105.

Liu S., Sjövall J. and Griffiths W. J. Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry. Anal.Chem. 75 (2003) 5835-5846.

Luisi S. Petraglia, F., Benedetto, C., Nappi, R.E., Bernardi, F., Fadalti, M., Reis, M., Luisi, M. and Genazzani, A.R. Serum allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patiens, J. Clin. Endocrin. Met. 85 (2008) 2429-2433.

Nieman L.K. Diagnostic Tests for Cushing's Syndrome. Ann. N.Y. Acad. Sci. 970 (2002) 112-118.

Majewska M. D., Harrison N. L., Schwartz R. D., Baker J. L. and Paul S. M. Steroid hormone metabolites and barbiturate-like modulators of the GABA receptor. Science 232 (1986) 1004-1007.

Mackenzie P.I, Owens I.S., Burchell B., Bock K.W., Bairoch A., Bélanger A., Fournel-Gigleux S., Green M., Hum D.W., Iyanagi T., Lancet D., Louisot P., Magdalou J., Chowdhury J.R., Ritter J.K., Tephly T.R., Schachter H., Tipton K.F. and Nebert, D.W. The UDP glycosyltransferase gene superfamily: Recommended update based on evolutionary divergence. Pharmacogenet. Genomics 7 (1997) 255-269.

Mackenzie P.I., Bock K.W., Burchell B., Guillemette C., Ikushiro S., Iyanagi T., Miners J.O., Owens I.S. and Nebert D.W. Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. Pharmacogenet. Genomics 10 (2005) 677-685.

Maeda N., Takana E., Suzuki S., Okumura K., Nomura S., Miyasho T., Haeno S., Yokota H. Accurate determination of tissue steroid hormones, precursors and conjugates in adult male rat. J. Biochem. 153(1) (2013) 63-71.

Marchi I., Rudaz S., Veuthey J-L. Atmospheric pressure photoionization for coupling liquid-chromatography to mass spectrometry: A review. Talanta 78 (2009) 1–18.

Marx C.E., Stevens R.D., Shampine L.J., Uzunova V., Trost W.T., Butterfield M.I., Massing M.W., Hamer R.M., Morrow, A.L. and Lieberman, J.A. Neuroactive steroids are altered in Schizoprenia and Bipolar disorders: Relevance to pathophysiology and therapeutics. Neuropsychopharmacol. 31(6) (2005) 1249-1263.

Matsui F., Koh E., Yamamoto K., Sugimoto K., Sin H-S., Maeda Y., Honma S., Namiki M. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of salivary testosterone and cortisol in healthy men for utilization in the diagnosis of late-onset hypogonadism in males. Endocr. J. 56(9) (2009) 1083-1093.

McNamara K.M., Harwood D.T., Simanainen U., Walters K.A., Jimenez M. and Handelsman D.J. Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography-tandem massspectrometry J. Steroid Biochem. Mol. Biol. 121 (2010) 611-618.

Mellon S.H. and Griffin L.D. Neurosteroids: Biochemistry and clinical significance. Trends Endocr. Met. 13 (2002) 35-43.

Meng L-J. and Sjövall J. Method for combined analysis of profiles of conjugated progesterone metabolites and bile acid in serum and urine of pregnant women. J. Chrom. B 688 (1997) 11-26.

Meng L-J., Reyes H., Palma J., Hernandez I., Ribalta J. and Sjövall J. Profiles of bile acids and pregesterone metabolites in the urine and serum of woman with intrahepatic cholestasis of pregnancy. J. Hepatol. 27 (1997), 346-357.

Mensah-Nyagan A.G., Do-Rego J-L., Beaujean D., Luu-The V., Pelletier G. and Vaudry H. Neurosteroids: Expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacol. Rev. 51 (1999) 63-81.

Milewich L., Gomez-Sanchez C., Madden J.D. and MacDonald P.C. Isolation and characterization of 5a-pregnane-3,20-dione and progesterone in peripheral blood of pregnant women. Measurement throughout pregnancy. Gynecol. Obstet. Invest. 6 (1970) 291-306.

Mitamura K., Yetera, Shimada K. Quantitative determination of pregnenolone 3-sulfate in rat brains using liquid chromatography/electrospray ionization-mass spectrometry. Anal. Sci. 15 (1999) 951-955.

Mitamura K., Nagaoka Y., Shimada K., Honma S., Namiki M., Koh E., Mizokami A. Simultaneous determination of androstenediol 3-sulphate and dehydroepiandrosterone sulfate in human serum using isotope diluted liquid chromatography-electrospray ionization-mass spectrometry. J. Chrom. B 796 (2003) 121-130.

Mojarrabi B., Butler R. and Mackenzie, P.I. cDNA cloning and characterization of the human UDP-glucuronosyltransferase, UGT1A3. Biochem. Biophys. Res. Commun. 225 (1996) 785-790.

Nathaniels P.W. Comparative studies on the initiation of labor. Eur. J. Obstet. Gynec. Reprod. Biol. 78 (1998) 127-132.

Nappi R.E, Petraglia F., Luisi S., Polatti F., Farina C. and Genazzani A.R. Serum allopregnanolone in women with postpartum 'blues'. Obstet. Gynec. 97 (2001) 77-80.

Radominska-Pandya A., Bratton S and Little J.M. A historical overview of the heterologous expression of mammalian UDP-glucuronosyltransferase isoforms over the past twenty years. Curr. Drug Metab. 6 (2005) 141-160.

Rauh M., Groeschl M., Rascher W. and Doerr H. G. Automated, fast and sensitive quantification of 17α-hydroxy-progesterone, androstenedione and testosterone by tandem mass spectrometry with on-line extraction. Steroids 71 (2006) 450-458.

Reddy M.I., Beotra A., Lal R.B., Khanduja R., Jain S., Kaur T. Analysis of glucocorticosteroids by atmospheric pressure chemical ionization-liquid chromatography mass spectrometry (APCI-LC/MS/MS) Indian J.Pharmacol. 39 (2007) 160-164.

Rupprecht R. Neuroactive steroids: mechanisms of action and neuropsychopharmacological properties. Psychoneuroendocr. 28 (2003) 139-168.

Rönquist-Nii Y. and Edlund P. O. Determination of corticosteroids in tissue samples by liquid chromatography-tandem mass spectrometry. J. Pharm. Biomed. Anal. 37(2) (2005) 341-350.

Paoletti A.M., Romagnino S., Rossana C., Orru M.M., Marotto M.F., Zedda P., Lello S., Biggio G., Concas A., Melis G.B. Observational study on the stability of the psychological status during normal pregnancy and increased blood levels of neuroactive steroids with GABA-A receptor agonist activity Psychoneuroendocr. 31 (2006), 485-492.

Parker C.R. Jr, Everett R.B., Quirk J.G. Jr, Whalley P.J., Gant N.F. Hormone production during pregnancy in the primigravid patient. I. Plasma levels of progesterone and 5-alpha-pregnane-3,20-dione throughout pregnancy of normal women and women who developed pregnancy-induced hypertension. Am. J. Obstet. Gynecol. 135 (1979) 778-782.

A. Pařízek, M. Hill, R. Kancheva, H. Havlíková, L. Kancheva, J. Cindr, A. Pašková, V. Pouzar, I. Černý, P. Drbohlav, Z. Hájek, L. Stárka, J. Clin. Endocr. Metab. Neuroactive pregnanolone isomers during pregnancy. J. Clin. Endocr. Metab. 90 (2005) 395-403.

Payne A.H. and Hales D.H. Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones. Endoc. Rev. 25(6) (2004) 947-970.

Pearson Murphy B.E., Steinberg S., Hu F.Y., Allison C.M. Neuroactive ring A-reduced metabolites of progesterone in human plasma during pregnancy: elevated levels of 5α-dihydroprogesterone in depressed patients during the latter half of pregnancy. J. Clin. Endocri. Metab. 86 (2001) 5981- 5987.

Pisu M. G. and Serra M. Neurosteroids and neuroactive drugs in metal disorders. Life Sci. 74 (2004) 3181-3197.

Pozo O.J., Van Eenoo P., Van Thuyne W., Deventer K. and Delbeke F.T. Direct quantification of steroid glucuronides in human urine by liquid chromatography-electrospray tandem mass spectrometry. J. Chromatogr A. 1183 (2008) 108-118.

Schwarz E., Liu A., Randall H., Haslip C., Keune F., Murray M., Longo N., Pasquali M. Use of steroid profiling by UPLC-MS/MS as a second Tier test in newborn screening for congenital adrenal Hyperplasia: The Utah experience. Pediatric Res. 66 (2009) 230-235.

Schumacher M., Weill-Engerer S., Liere P., Robel F., Franklin M., Garcia-Segure L.M., Lambert J.J., Mayo W., Melcangi R.C., Parducz A., Suter U., Carelli C. and Baulieu E.E. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Progr. Neurobiol. 71 (2003) 3-29.

Schumacher M., Liere P., Akwa Y., Rajkowski K., Griffits W., Bodin K., Sjövall J. and Baulieu, E.E. Pregnenolone sulfate in the brain: a controversial neurosteroid. Neurochem. Int. 52 (2008) 522-540.

Sakakibara Y., Suiko M., Pai T.G, Nakayama T., Takami Y., Katafuchi J. and Liu M.C. Highly conserved mouse and human brain sulfotransferases: molecular cloning, expression, and functional characterization. Gene 285 (2002) 39-47.

Shackleton C.H.L. Profiling steroid hormones and urinary steroids. J. Chrom. 379 (1986) 91-156.

Shimada K., Xie F., Niwa T.,Wakasawa T. and Nambara T. Studies on steroids: CCXXIX. Separation and characterization of catechol oestrogen glucuronides in urine of pregnant women by high-performance liquid chromatography J. Chrom. A 400 (1987) 215-221.

Starlard-Davenport A., Lyn-Cook B., and Radominska-Pandya A. Novel identification of UDP-glucuronosyltransferase 1A10 as an estrogen-regulated target gene. Steroids 73 (2008) 139-147.

Stener-Victorin E., Holm G. Labrie f., Nilsson,L., Janson P.O and Ohlsson C. Are there any sensitive and Specific sex steroid markers for Polycystic ovary Syndrome? J. Clin. Endocr. Metab. 95(2010) 810-81.

Stoa K.F. and Bessesen A. Identification and quantitation of 5a-pregnanedione in human pregnancy plasma. J. Steroid Biochem. 6 (1975) 21-25.

Stoffel-Wagner B. Clinical study: neurosteroid metabolism in the human brain. Eur. J Endocr. 145 (2001) 669-679.

Stoffel-Wagner B. Neurosteroid biosynthesis in the human brain and its clinical implications. Ann. N.Y. Acad. Sci. 1007 (2003) 64-78.

Strahm E., Kohler I., Rudaz S., Martel S., Carrupt P.A., Veuthey J-L., Saugy M. and Saudan C. Isolation and quantification by high-performance liquid chromatography-ion-trap mass spectrometry of androgen sulfoconjugates in human urine. J. Chrom. A 1196-1197 (2008) 153-160.

Strassburg C.P., Manns M.P. and Tukey R.H. Expression of the UDP-glucuronosyltransferase 1A locus in human colon. Identification and characterization of the novel extrahepatic UGT1A8. J. Biol. Chem. 273 (1998) 8719-8726.

Suleman F.G., Abid A., Gradinaru D, Daval J-L., Magdalou J., Minn A. Identification of the uridine diphosphate glucuronosyltransferase isoform UGT1A6 in rat brain and in primary cultures of neurons and astrocytes. Arch. Biochem. Biophys. 358(1998) 63-7.

Taieb J., Mathian B., Millot F., Patricot M-C., Mathieu E., Queyrel N., Lacroix I., Somma-Delpero C. and Boudou P. Testosterone measured by 10 immunoassays and by isotope dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. Clin. Chem. 49 (2003) 1381-1395.

Taylor R. L., Machacek, D. and Singh R. J. Validation of a high-throughput liquid chromatography-tandem mass spectrometry method for urinary cortisol and cortisone. Clin. Chem. 48 (2002) 1511-1519.

Tsutsui S., Ukena K., Usui M., Sakamoto H. and Takase M. Novel brain function: biosynthesis and actions of neurosteroids in neurons. Neurosci. Res. 36 (2000) 261-273.

Tukey R.H. and Strassburg C.P. Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. Annu. Rev. Pharmacol. Toxicol. 40 (2000) 581-616.

Turgeon D., Carrier J.S., Lévesque É., Hum D.W. and Bélanger A. Relative enzymatic activity, protein stability, and tissue distribution of human steroid-metabolizing UGT2B subfamily members. Endocr. 142 (2001) 778-787.

Valleé M., Rivera J.D., Koob G.F., Purdy R.H. and Fitzgerald R.L. Quantification of neurosteroid in rat plasma and brain following swin sress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry. Anal. Biochem. 287 (**2000**) 153-166.

Wang C. and Gardinali P.R. Comparison of multiple API techniques for the simultaneous detection of microconstituents in water by on-line SPE-LC-MS/MS. J. Mass Spectrom. 47 (2012) 1255-1268.

Weill-Engerer S., David J-P., Sazdovitch V., Liere P., Eyechenne B., Pianos A., Schumacher M., Delacourte A., Baulieu E.E. and Akwa Y. Neurosteroid quantification in human brain regions: Comparison between Alzheimer,s and nondemented patients. J. Clin. Endocr. Metab. 87 (2002) 5138-5143.

Weidong D., Qiang H., Peiyuan Y., Jia L., Jia Z., Hongwei K., Chinxia Z., Xin L. Guowang X. Comprehensive and highly sensitive urinary steroid hormone profiling method base on stable isotope-labelling liquid chromatography-mass spectrometry. Anal. Chem. 84 (2012) 10245-10251.

Wu W.X., Ma X.H., Coksaygan T., Chakrabarty K., Collins V., Rose J. and Nathaniels P.W. Prostaglandin mediates premature delivery in pregnant sheep induced by estradiol at 121 days of gestational age. Endocr. 145 (2004) 1444-1452.

Xu X., Roman J.M., Issaq H.J., Keefer L.K., Veenstra T.D. and Ziegler R.G. Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. Anal. Chem. 79 (2007) 7813-7821.

Yamashita K., Takahashi M., Tsukamoto S., Numazawa M., Okuyama M. and Honma S. Use of novel piconolinoyl derivatization for simulation quantification of six corticosteroids by liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chrom. A 1173 (2007) 120-128.

Yang Y.J., Lee J., Choi M.H. and Chung B.C. Direct determination of estriol 3- and 16-glucuronides in pregnancy urine by liquid chromatography with electrospray. Biomed. Chromatogr. 17 (2003) 219-225.

Yu L., Romero D.G., Gomez-Sanchez, C.E. and Gomez-Sanchez E.P. Steroidogenic enzyme gene expression in the human brain. Mol. Cell Endocr. 190 (2002) 9-17**.**

You L. Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. Chem.--Biol. Interact. 147 (2004) 233-246.

Zhang F., Rick D.L., Kan L. H., Perala A.W., Geter D.R., Lebaron M.J. and Bartels M.J. Simultaneous quantitation of testosterone and estradiol in human cell line (H295R) by

liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. Rapid Com. Mass Spectrom. 25 (2011) 3123-3130.

Zhang W., Liu W., Innocenti F. and Ratain M.J. Searching for tissue specific expression pattern-linked nucleotides of UGT1A isoforms. 2 (2007) PLoS ONE e396. doi:10.1371/journal.pone.0000396