Mass Spectrometry Based Determination of Unconjugated, Sulfated and Glucuronidated Steroid Hormones and Their Metabolites in Biological Fluids

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List of Abbreviations (according to the order of appearance)

Acetyl-CoA	Acetyl-coenzyme A
DHEA	Dehydroepiandrosterone
T	Testosterone
4A	4-Androstenedione
DHT	Dihydrotestosterone
CYP	Cytochrome P450
CYP11A1	Cholesterol 20,22-desmolase
CYP3A4/5	16α-hydroxylase
CYP17A1	17α-hydroxylase
CYP11B1	11β-hydroxylase
CYP11B2	Aldosterone synthase
17βHSD	17β-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
SRD5A	5α-reductase
E1	Estrone
E2	Estradiol
E3	Estriol
PAPS	3′-phosphoadenosine-5′-phosphosulfate
APS	Adenosine-5′-phosphosulfate
ATP	Adenosine triphosphate
STS	Sulfatase
ADP	Adenosine diphosphate
PAP	3′-phospho-adenosine-5′-phosphate
UGTs	Uridine diphosphoglucuronosyltransferase
UDPGA	Uridine 5'-diphosphoglucuronic acid
UTP	Uridine triphosphate

PP_i	Inorganic pyrophosphate
UDP	Uridine Diphosphate
NADH	Nicotinamide adenine dinucleotide
NAD+	Nicotinamide adenine dinucleotide ion
DHEAS	Dehydroepiandrosterone sulfate
16α-OH-DHEAS	16α-hydroxydehydroepiandrosterone sulfate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LC	Liquid chromatography
MS	Mass spectrometry
MeOH	Methanol
ACN	Acetonitrile
HPLC	High performance liquid chromatography
UV/Vis	Ultraviolet/Visible
PDA	Photodiode array
GC	Gas chromatography
m/z	Mass-to-charge
APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization
RF	Radio frequency
DC	Direct current
CID	Collision-induced dissociation
MRM	Multiple reaction monitoring
GC-MS	Gas chromatography-mass spectrometry
EI	Electron impact
LLE	Liquid-liquid extraction
SPE	Solid phase extraction
LLOQ	Lower limit of quantification

AF	Amniotic fluid
FDA	Food and Drug Administration
QC	Quality control
SD	Standard deviation
E3S	Estriol sulfate
PD	Pregnanediol
THE	Tetrahydrocortisone
P5-3β,20α,21-triol	5-pregnene-3β,20α,21-triol
21-OH-P5olon	5-pregnene-3β,21-diol-20-one
16α−OH-P5olon	5-pregnene-3β,16α-diol-20-one
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
An-G	Androsterone glucuronide
Etio-G	Etiocholanolone glucuronide
epiAn-G	Epiandrosterone glucuronide
DHT-G	Dihydrotestosterone glucuronide
DHEA-G	Dehydroepiandrosterone glucuronide
T-G	Testosterone glucuronide
epiT-G	Epitestosterone glucuronide
E2-17G	17β-estradiol 17-glucuronide
E2-3G	17β-estradiol 3-glucuronide

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1 Introduction

1.1 Steroids

1.1.1 Steroid classification and biosynthesis

Steroids are small biologically active molecules with a distinctive chemical structure of seventeen carbon atoms arranged in 4 condensed rings: three cyclohexanes (ring A, B and C) and one cyclopentane (ring D). The 4-ring skeleton is also called cyclopentanoperhydrophenanthrene (Figure 1a) (Gomes et al., 2009). Steroids are ubiquitously present in nature, e.g., animals, plants, and fungi. Steroids play significant roles as signaling molecules and constituents of cell membranes.

Cholesterol (Figure 1b) (Moss, 1989), as an essential component of cell membranes, influences their integrity, fluidity, and permeability (Chatuphonprasert et al., 2018). Its presence has been widely reported in the body tissues of vertebrates and is especially found in high concentrations in brain, liver, and spinal cord. Its biosynthesis requires various enzymes via a complex route beginning with the condensation of two acetyl-coenzyme A (acetyl-CoA) (Olivera et al., 2019). In addition, cholesterol is the principal precursor of vitamin D, bile acids as well as other steroid hormones. Steroid hormones can be classified according to their biologically activities into progestogens, androgens, estrogens, glucocorticoids and mineralocorticoids.

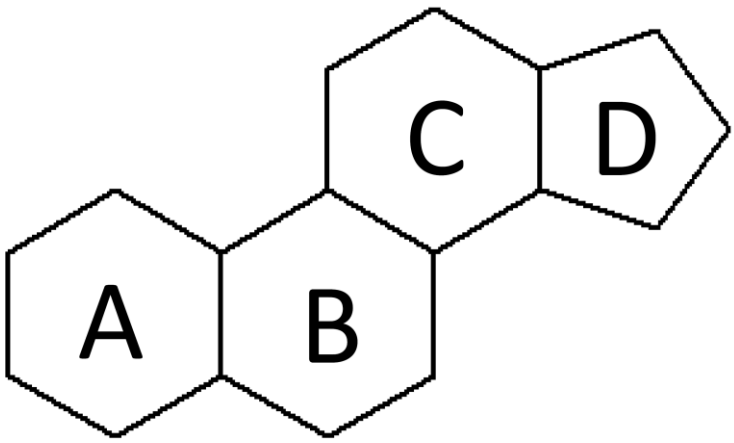


Figure 1a. Cyclopentanoperhydrophenanthrene

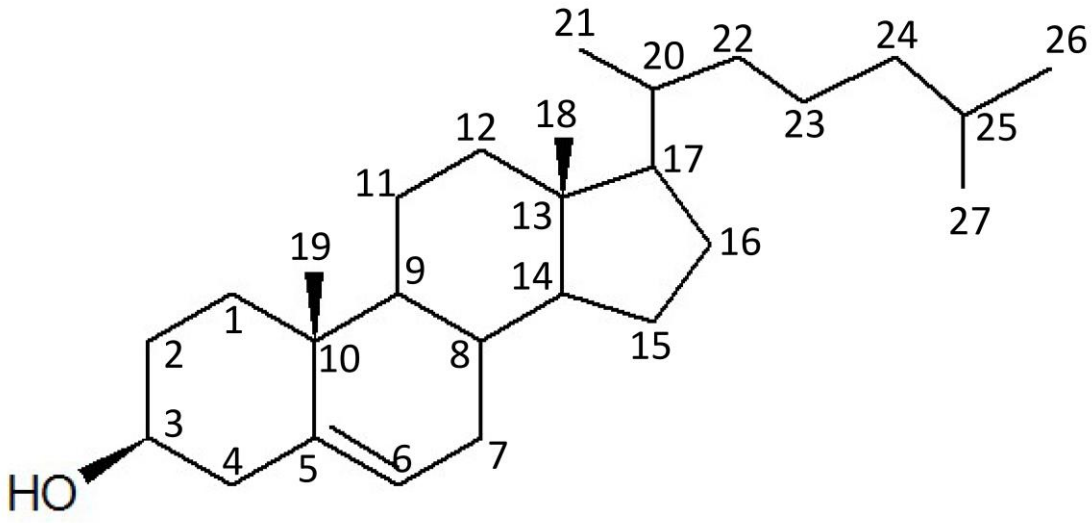


Figure 1b. Cholesterol and carbon numbering

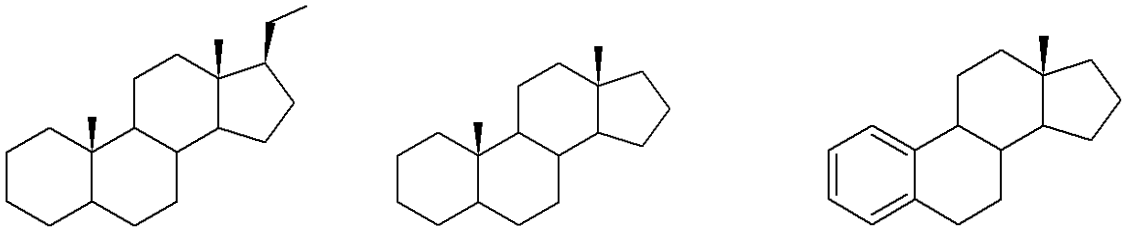
Progestogens (progestagens or gestagens) are characterized by a 21-carbon skeleton termed as "pregnane skeleton" (Figure 2). Together with androgens and estrogens, they constitute three types of sex hormones. As showed in Figure 3, cholesterol can be converted to pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A). Pregnenolone is then the substrate for the biosynthesis of progesterone, which is the most important and major progestogen in the body. The biological function of progestogens is the regulation of the female reproductive system, such as the menstrual cycle and the pregnancy process (Schiffer et al., 2019). Progestins are synthetic progestogens and are used for maintaining early stages of pregnancy, amenorrhea, and irregular menstrual bleeding in medicine (Clark et al., 2011).

Androgens are composed of a 19-carbon skeleton which is called an androstane skeleton (Figure 2). Androgens are involved mainly in male sex development by binding androgen receptors. Androgens are mainly synthesized in testes, ovaries and adrenal glands. Androgens synthesized in the adrenal cortex are adrenal androgens. They include dehydroepiandrosterone (DHEA), testosterone (T), androstenedione (4A) and dihydrotestosterone (DHT) (Rainey et al., 2004).

Estrogens have a skeleton of 18-carbon molecules with an aromatic A-ring, known as an estrane skeleton (Figure 2). Estrogens play a significant role in the development and regulation of the female reproductive system (Robinson et al., 1977). There are three

major endogenous estrogens: estrone (E1), estradiol (E2) and estriol (E3). Their biosynthesis happens in all vertebrates and some insects.

Corticoids are also steroids with 21 carbons, characterized by a hydroxyl group at position 21. Glucocorticoids and mineralocorticoids are the two main types of corticosteroids (Figure 3). Cortisol is the most important glucocorticoid, while aldosterone is the crucial mineralocorticoid. They are involved in the regulation of carbohydrate metabolism, stress mechanisms, immune system, cardiovascular circulation and blood electrolyte levels (Williams, 2018).



Pregnane skeleton (C21) Androstane skeleton (C19) Estrane skeleton (C18)

Figure 2. The structural classification of steroids.

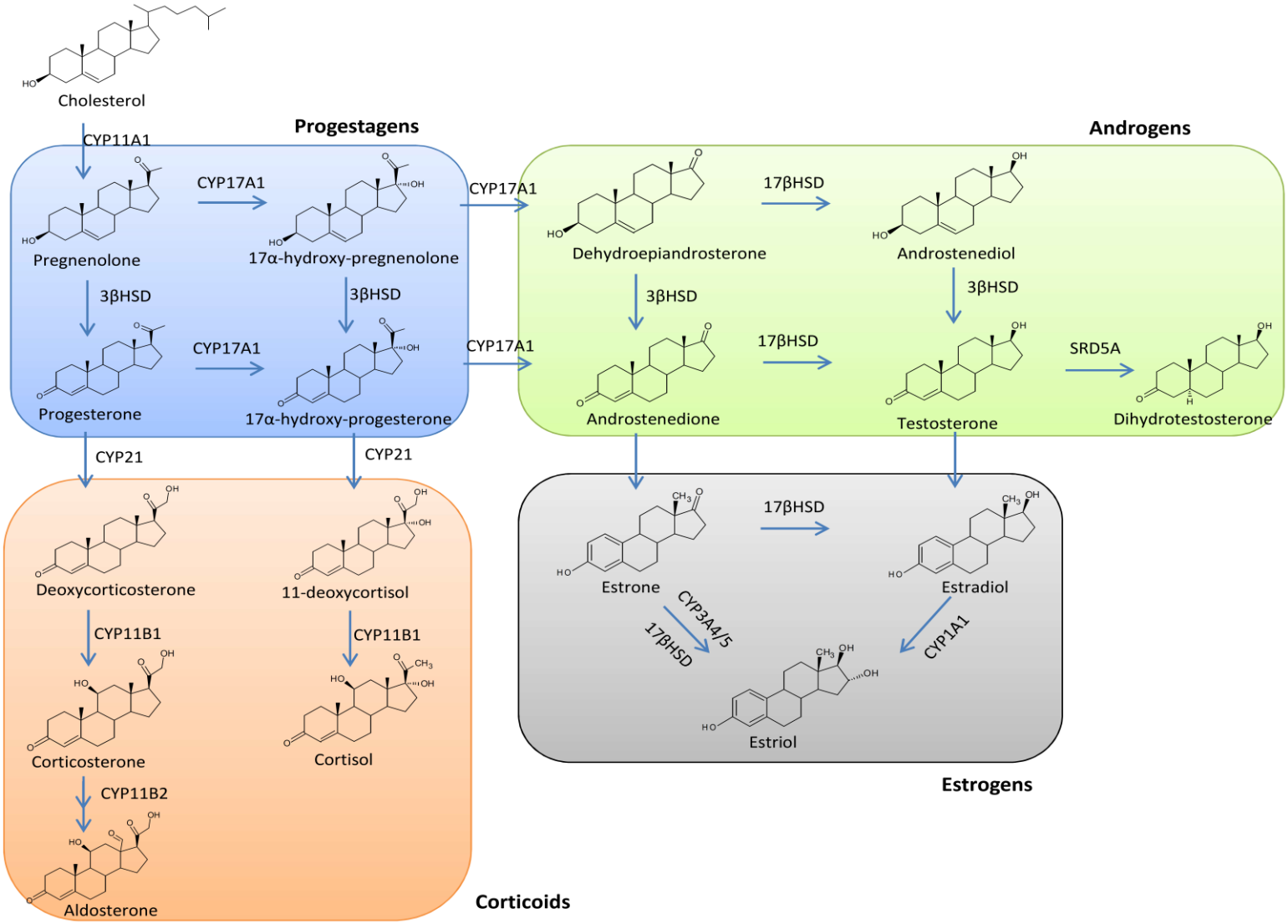


Figure 3. Steroid biosynthesis in the human being (modified according to Schiffer et al., 2019).

1.1.2 Steroid Conjugates and biosynthesis

Steroid hormones are synthesized by human endocrine glands and then secreted into the blood circulation. The metabolism of steroid hormones consists of phase I metabolism and phase II metabolism. Phase I metabolism refers to oxidation, reduction and hydrolysis of functional groups (e.g. hydroxyl or ketone groups) attached to the steroid backbone at different positions (Pranata et al., 2019). Phase II metabolism involves conjugation reactions with a charged moiety, either a sulfate group or a glucuronic acid group (Jaentti et al., 2006). Steroids are predominantly present as conjugated forms in human urine and blood (Mcnamara et al., 2013).

The addition of sulfated and glucuronidated moieties to steroid backbones increases the solubility of steroids. Therefore, conjugation is considered to be a pathway for excretion. The main organ for steroid inactivation and catabolism is liver, but this process can also take place in the kidney (Bélanger et al., 2003) (Mueller et al., 2015). Figure 4 exemplifies the sulfated and glucuronidated conjugation reactions of androsterone, a major steroid in human blood and urine.

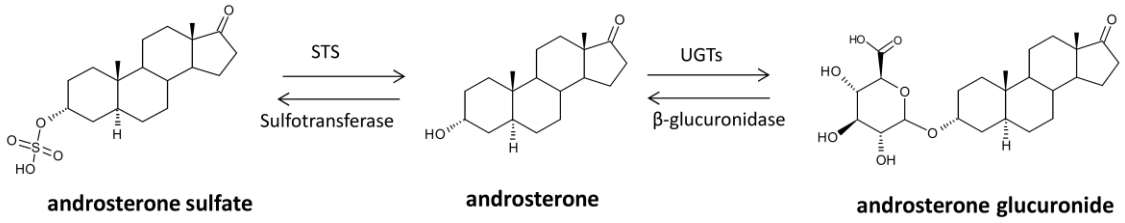


Figure 4. Sulfated and glucuronidated conjugation reactions of androsterone (STS, steroid sulfatase; UGTs, uridine diphosphoglucuronosyl transferase).

For a long time, the biological role of conjugated steroids remained less understood. It has been believed that only unconjugated steroids could pass through cell membranes to interact with receptors and then exert biological effects (Mueller et al., 2015). However, recent studies have found that conjugated steroids can be transported into cells by specific transporters: for example, sodium-dependent organic anion transporter (SOAT) can transport biologically inactive sulfated steroids into specific cells, where they are reactivated by the steroid sulfatase (STS) to biologically active unconjugated steroids (Bakhaus et al., 2017) (Geyer et al., 2017). Androgen glucuronides can be deconjugated by multidrug resistance protein (MRP) in human gut lumen (Li et al., 2019). MRP3 can

transport estrone glucuronide (E1-3G), estradiol 3-glucuronide (E2-3G), estriol 3-glucuronides (E3-3G) and estriol 16-glucuronides (E3-16G) with rather high affinity, whereas MRP4 can only transport E3-16G in the vesicular transport assay performed in vitro (Jaervinen et al., 2017).

Formation of steroid sulfates (Figure 5) is catalyzed by sulfotransferase, which transfers the sulfate group of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to available steroid hydroxyl groups (Hobkirk, 1985) (Hobkirk, 1993). The sulfate donor PAPS derives from phosphorylation of adenosine-5′-phosphosulfate (APS). APS arises from adenosine triphosphate (ATP) by ATP-sulfate adenylytransferase. On the other hand, sulfated steroids are desulfated by steroid sulfatase (STS) to free steroids which are considered the active forms.

The mechanism of steroid glucuronidation is depicted in Figure 6. Uridine diphosphoglucuronosyl transferase (UGTs) is the enzyme transferring the glucuronyl group from uridine 5'-diphosphoglucuronic acid (UDPGA) to steroids (Argikar, 2012). UDPGA is synthesized from uridine diphosphate-glucose (UDP-glucose, UDPG) and Nicotinamide adenine dinucleotide ion (NAD^+) by UDPG dehydrogenase. Phosphorylase catalyzes the production of UDP-glucose from glucose-1′-phosphate and uridine triphosphate (UTP). In contrast, β-glucuronidase catalyzes the hydrolysis of steroid glucuronides by cleaving the β-glucuronides.

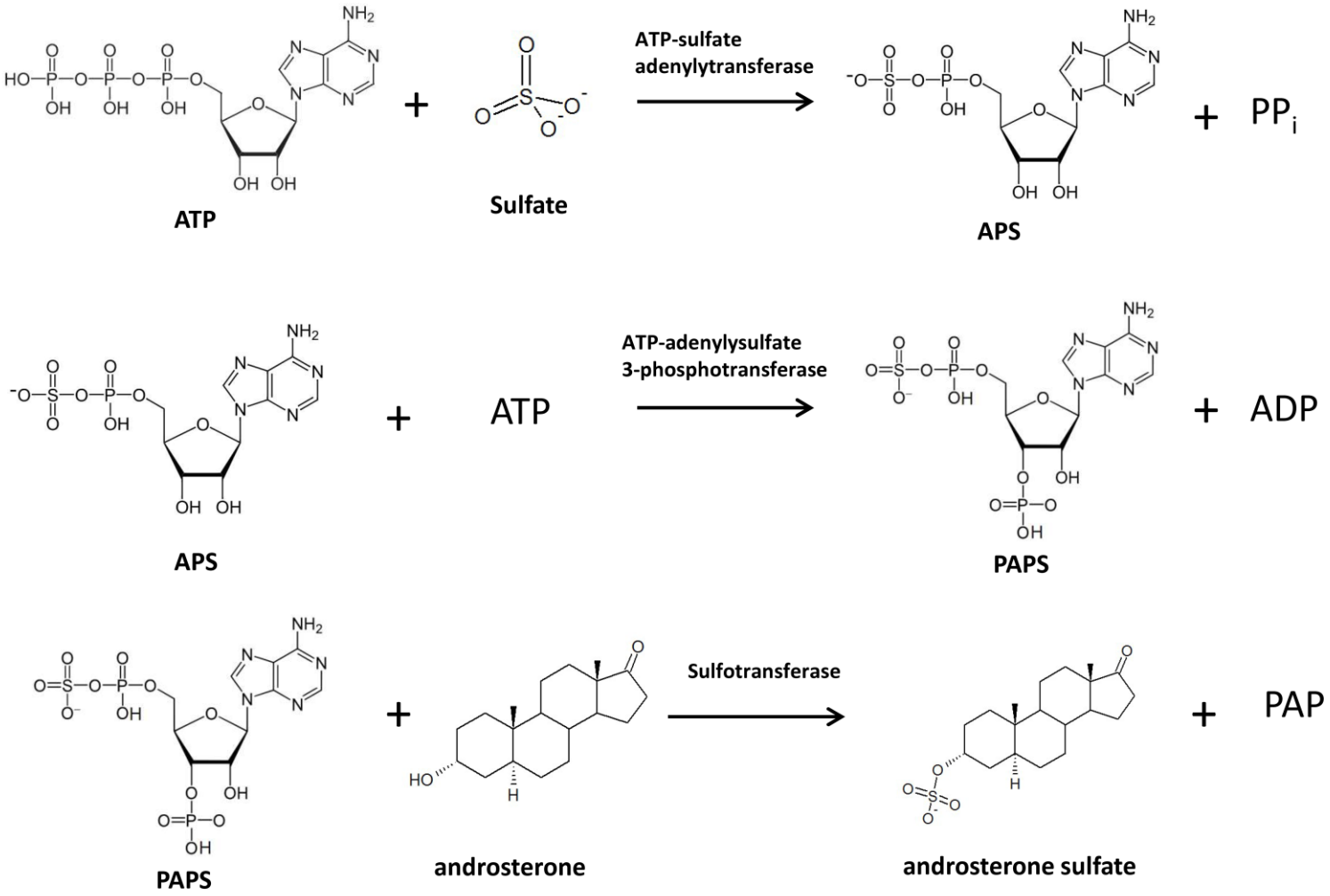


Figure 5. Reaction mechanism of sulfation (ATP, adenosine triphosphate; PP_i, inorganic pyrophosphate; APS, adenosine-5′-phosphosulfate; ADP, adenosine diphosphate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PAP, phosphoadenosine phosphate).

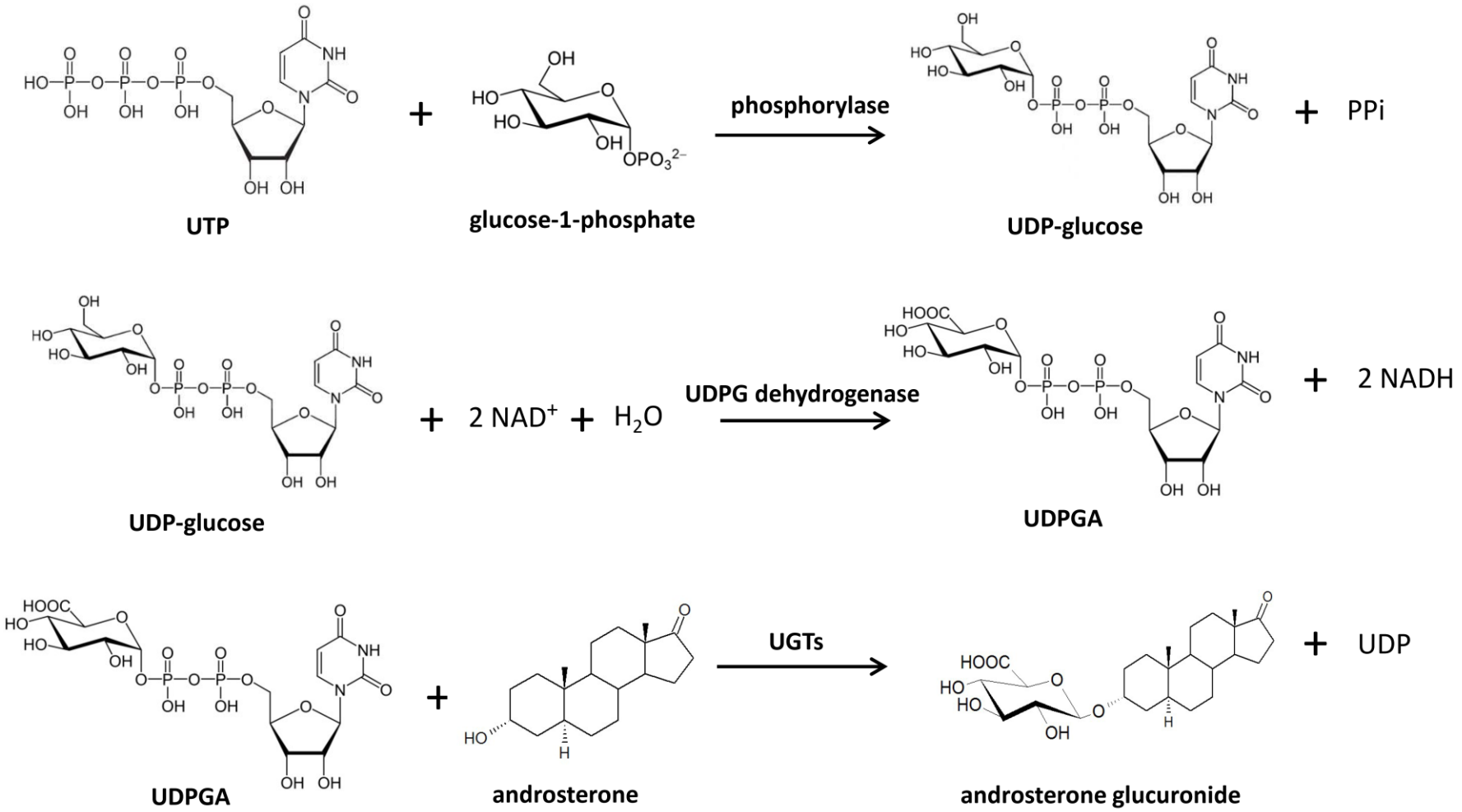


Figure 6. Reaction mechanism of glucuronidation (UTP, uridine triphosphate; PP_i, inorganic pyrophosphate; UDP-glucose, uridine diphosphate glucose; NAD+/NADH, oxidized/reduced nicotinamide adeninedinucleotide; UDPGA, uridine 5'-diphosphoglucuronic acid; UDP, uridine diphosphate) (Wang et al., 2021).

1.1.3 Adrenal steroidogenesis

1.1.3.1 Adrenal steroidogenesis in fetus

During the development of the human fetus, the fetal adrenal glands appear around one month after fertilization and then reach their maximum size relative to their adult counterparts by the fourth fetal month (Morel et al., 2016) (Shackleton, 1984). The fetal adrenal glands have large capacity to produce steroids (Ishimoto et al., 2011). The fetal adrenal gland differentiates into three zones from human adrenal anlage by the eighth week of pregnancy:

1) An outer adult (permanent) zone: The adult zone remains unchanged during pregnancy. The adult zone grows to form the adult glands after birth. The adult zone is responsible for the production of mineralocorticoids.

2) A transitional zone: The transitional zone exists between the adult zone and fetal zone. The transitional zone of the fetal adrenal glands is believed to be the site of fetal *de novo* cortisol production from the second half of gestation onwards. The transitional zone expresses 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) after the 28th week of gestation which allows the conversion of pregnenolone into 17α-OH-progesterone. Thereafter, cortisol is synthesized through 21-hydroxylation and 11β-hydroxylation (Figure 3).

3) An inner fetal (transient) zone: The fetal zone increases the size of volume and production of steroids as gestation progresses (Heckmann et al., 2006). However, the fetal zone gradually disappears several months after delivery. The fetal zone secretes tremendous amounts of sulfated Δ5-steroids. Large amounts of DHEA sulfate (DHEAS) are synthesized by17α-hydroxylase/17-20 lyase in the fetal adrenal glands. DHEAS is then metabolized to 16α-hydroxydehydroepiandrosterone sulfate (16α-OH-DHEAS) by 16α-hydroxylase in fetal liver (Wang et al., 2019).

1.1.3.2 Adrenal steroidogenesis in feto-placental unit

In humans, the fetus develops in the uterus of the mother. The placenta connects the developing fetus to the uterus of the mother via the umbilical cord. This system is known as the feto-placental unit (Mayo, 2018). The synthesis and metabolism of steroids in the feto-placental unit is quite complicated but important. Due to the difference in activity of steroid metabolism between mother, fetus and placenta, the steroids synthesized in each of the three compartments are not the same. Figure 7 shows the steroid metabolism pathways in the feto-placental unit.

1) In the fetal compartment, the fetal adrenal glands cannot synthesize progesterone from pregnenolone since it lacks 3β-hydroxysteroid dehydrogenase (3β-HSD) (Nussdorfer, 1985).

2) In the placental compartment, pregnenolone is the main metabolite of cholesterol. The lack of 17α-hydroxylase/17, 20-lyase in the placenta leads to its incapability to produce androgens by itself. Instead, 16α-OH-DHEAS is metabolized to 16α-OH-DHEA by sulfatase which afterwards is aromatized to E3.

3) In the maternal compartment, steroids synthesized by the mother, can cross the placenta to enter the fetal compartment.

1.1.3.3 Adrenal steroidogenesis in adults

In adults, the human adrenal gland consists of the inner medulla and surrounding cortex. The medulla produces hormones such as adrenaline and noradrenaline. The cortex is formed by three different layers (Greaves et al., 2018).

1) Zona glomerulosa: the outermost layer zone secretes mineralocorticoids, especially aldosterone, which regulates salt and water balance.

2) Zona fasciculata: the middle layer zone produces glucocorticoids, principally cortisol, which maintains glucose homeostasis.

3) Zona reticularis: the innermost layer zone is responsible for the production of androgens, predominantly DHEA/DHEAS. DHEA/DHEAS are neuroactive neurosteroids and their secretion is under the control of adrenocorticotropic hormone (ACTH). DHEAS is the most abundant endogenous steroid circulating in micro molar concentrations in blood (Campbell, 2020). DHEAS is converted to DHEA by sulfatase. However, the levels of DHEA/DHEAS vary over lifetime (Remer et al., 2005). They remain in low concentrations during childhood until adrenarche, defined as the onset of increased production of DHEA/DHEAS by the adrenal cortex between age 6 and 8 years. At this point, the levels of DHEA/DHEAS markedly increase and peak at between 20 and 30 years of age. Afterwards, DHEA and DHEA-S levels decrease gradually. DHEA/DHEAS are the precursors of other androgens and estrogens.

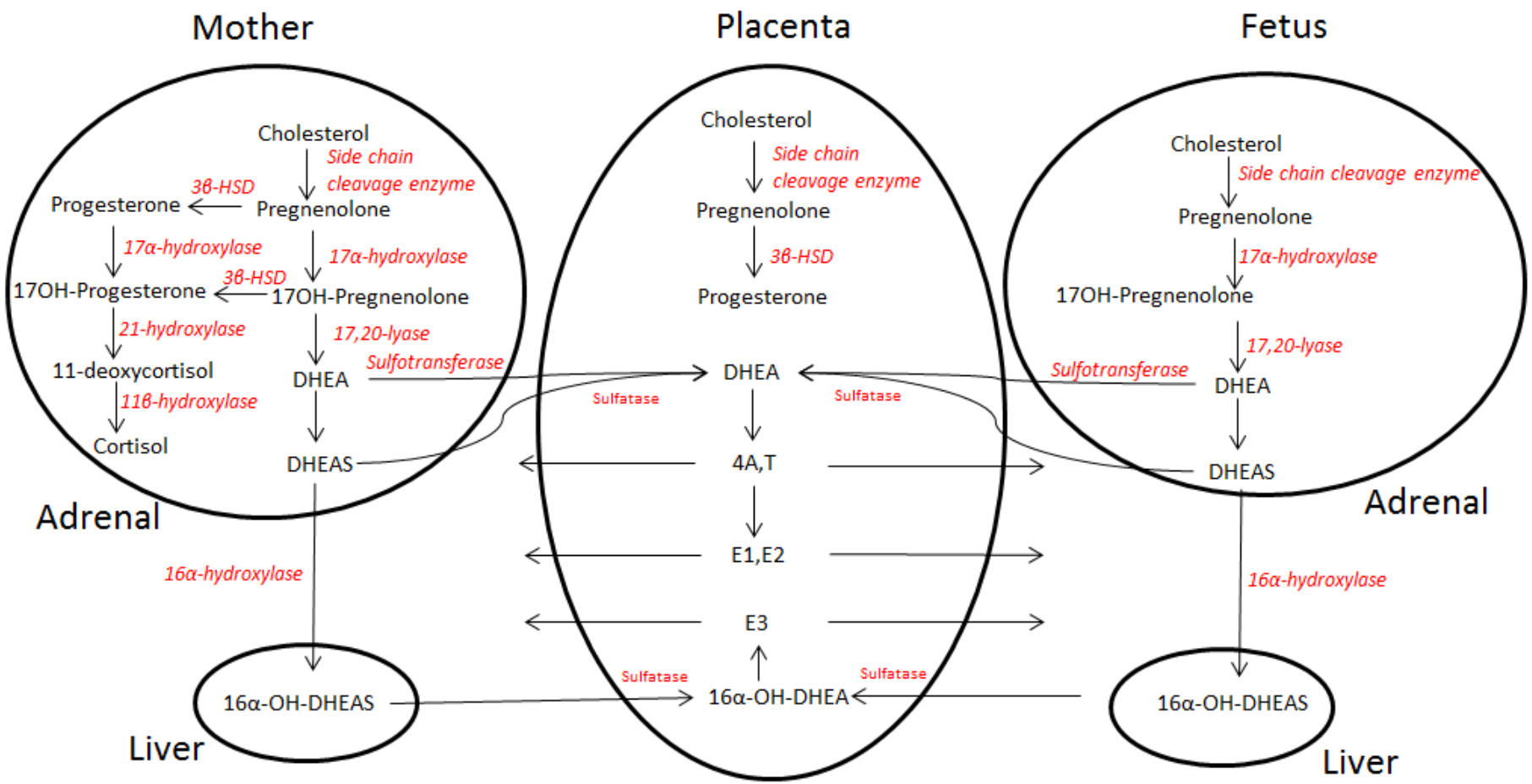


Figure 7. Steroid metabolism and enzymes in the feto-placental unit (Wang et al., 2019).

1.2 Mass spectrometry

1.2.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

1.2.1.1 Liquid chromatography (LC)

Chromatography is a separation technique to effectively isolate compounds from a mixture. The difference in physicochemical properties (e.g. adsorption capacity, solubility, molecular size, molecular polarity, affinity, charge) of compounds in a mixture lead to different distribution coefficients between the stationary phase and the mobile phase. A stationary phase usually consists of small particles which are packed into a glass or metal tube. A mobile phase is usually a liquid or gas which flows through the stationary phase. When analytes move with the mobile phase flow through the stationary phase, the compounds with a stronger affinity to the stationary phase will move more slowly due to longer interaction time. The retention time is defined as the time of an analyte staying on the column. The chromatogram is a plot of retention times of analytes against detector responses of analytes. X axis represents the retention time; Y axis stands for the response intensity. The area under the peak is often used for quantification (Figure 8).

Liquid chromatography refers to a type of chromatography using liquid as the mobile phase. The goal for analytical LC is to identify and perform qualitative and quantitative analysis of the compounds.

Based on the types of stationary phases and mobile phases, there are several types of LC. The most representative variants are normal phase chromatography and reverse phase chromatography, both of which are widely applied in the analysis of food, pharmaceutical and clinical fields (Engel et al., 2019) (Romand et al., 2016). In normal phase chromatography, the polarity of the stationary phase is higher than that of the mobile phase. Silica gel is the common stationary phase for the retention of polar compounds. Organic solvents such as hexane and chloroform are often used as mobile phases. Conversely, reverse phase chromatography employs non-polar stationary phase as the stationary phase (e.g. C8, C18, C30) while the mobile phase is polar solvents (e.g. MeOH, ACN, water) (Adaway et al., 2015).

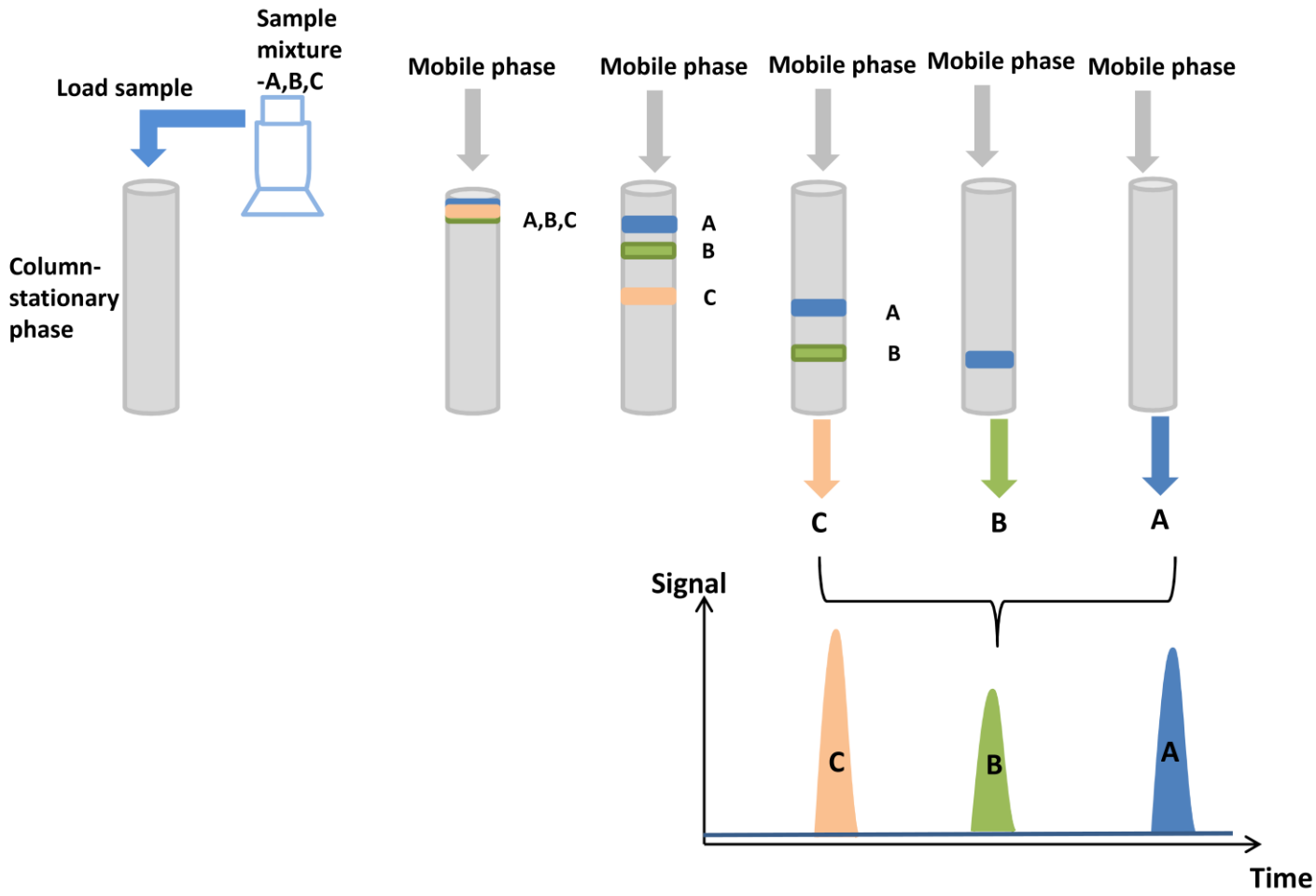


Figure 8. Schematic diagram of chromatography. Sample mixture containing A, B and C are loaded on the column and eluted by mobile phase according to their partition coefficient.

The operational pressure of the LC system forcing the mobile phase to pass through the column depends on the force of gravity. High performance liquid chromatography (HPLC) applies much higher pressure to the system, and allows much smaller particle size (2-10 μm) and inner diameter (2.1-4.6 mm) of the analytical column. A HPLC instrument typically includes a sampler, a column, pumps, a degasser, a detector and a computer. The sampler brings the sample to the flow of mobile phase and introduces it onto the column. The pumps are responsible to deliver the correct flow of mobile phase to the column. The degasser removes bubbles in the mobile phase. The detector transforms the amount of compounds flowing out from the column into signals. The computer controls the entire instrument system and provides data analysis. Commonly used detectors are UV/vis, photodiode array (PDA) or mass spectrometer.

1.2.1.2 Mass spectrometry (MS)

MS is a sensitive technique for the detection, identification, and quantification of molecules by measuring mass-to-charge (m/z) ratios of molecules. It is universally applied to analyze a wide range of biological molecules, including hydrophilic and hydrophobic molecules. Nowadays, MS has become one of the most powerful analytical techniques due to its high selectivity (specificity) and sensitivity and is often coupled to other chromatographic techniques such as LC or gas chromatography (GC). The development of LC-MS was started in the 1970s, capillaries was first used to connect LC columns and MS ion sources (Tal'roze et al., 1978).

The typical mass spectrometer consists of an ion source, a mass analyzer and a detector (Ardrey, 2003).

In a mass spectrometer, samples first enter the ion source by a sample introduction system. The molecules in samples are then ionized to molecular and fragment ions with different m/z ratios based on their different structures. The ions are accelerated to enter the mass analyzer which separates the ions by their m/z ratios. In a time-of-flight mass spectrometer, the ions with lower m/z ratios will travel faster and reach the detector earlier than the ions with higher m/z ratios. The detector records the ions' signal. A mass chromatogram is a chromatogram for specific defined mass data. Usually the X-axis represents time and the Y-axis represents signal intensity.

Ion source

The ion source in LC-MS/MS is a device that generates ions from the analytes which had been already separated by LC. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are the main types of ionization in LC-MS/MS. Both of them ionize samples at atmospheric pressure (Gomes et al., 2009).

As a soft ionization, ESI (Figure 9a) is capable of interfacing between LC, which operates under atmospheric pressure, and a mass analyzer, which operates under vacuum. Figure 9a shows mechanism of ESI in positive mode. During electrospray, compounds in liquid phase are transformed into the aerosol. First, the sample flow enters the ESI nozzle and is sprayed into a strong positive electric field (high voltage: +3kV-+5kV) with the assistance of a flow of nitrogen. A Taylor cone is formed at the end of needle because of the high voltage, which helps to generate droplets. The solvent evaporates from the positive charged droplets until the density of charges increases to a limit (Rayleigh limit) at which the Coulomb repulsion force of charges offsets the surface tension of droplets, a Coulomb explosion takes place which generates smaller charged droplets. A warm dry nitrogen stream expels the solvent from charged droplets. This process is repeated continuously until all analytes are ionized and in gas phase. In negative mode, the negative voltage (-3kV--5kV) is applied to the needle. Negative charged droplets are formed which leads to generate negative ions.

APCI (Figure 9b) is also considered to be a soft ionization process but not as soft as ESI, it operates at high temperature. Droplets are formed with the assistance of sheath and auxiliary gases. As droplets travel through a heated vaporizer tube, the solvents and samples become vaporized. High voltage is applied to a Corona discharge needle to generate solvent ions. These solvent ions react with analytes' molecules in the gas phase to form ions of analytes in the gas phase.

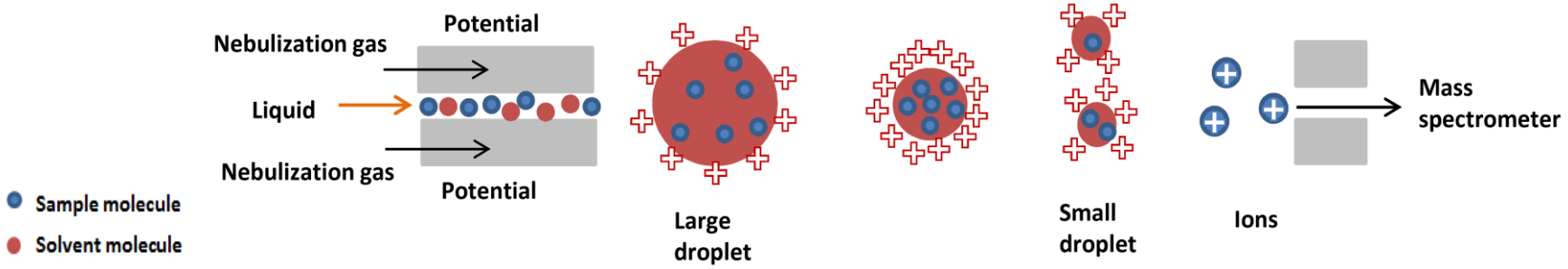


Figure 9a. Schematic representation of ESI ion source.

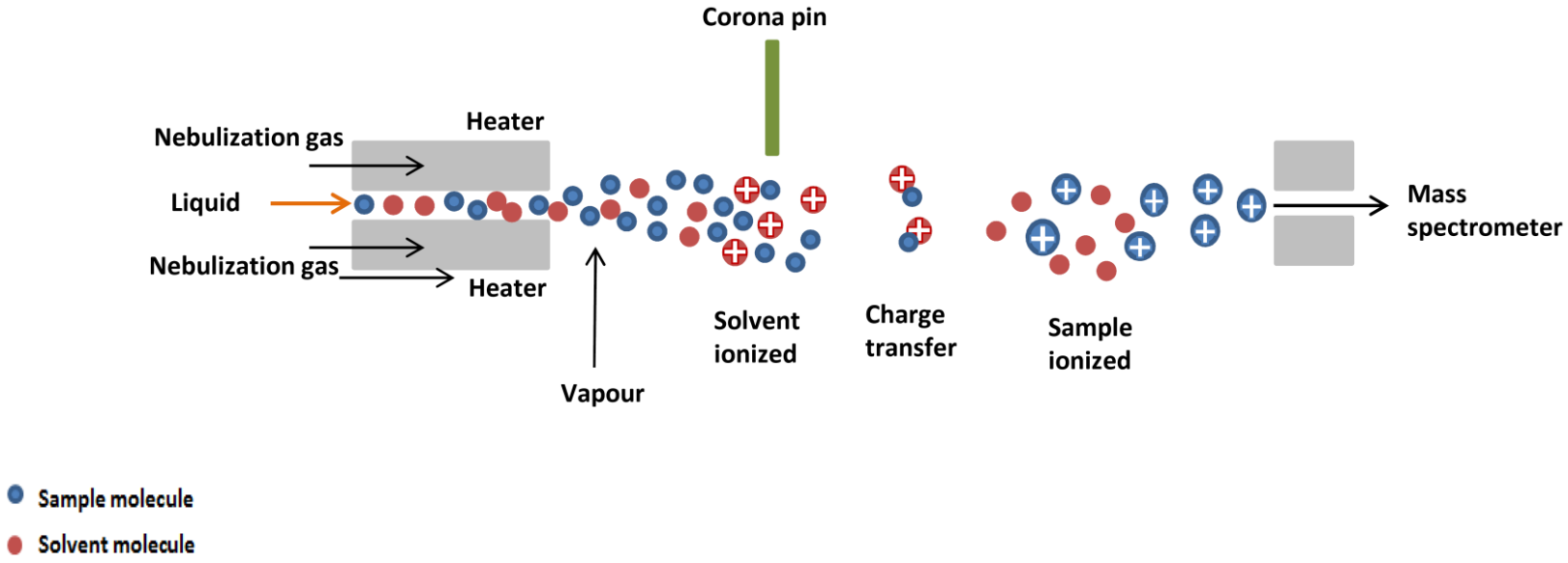


Figure 9b. Schematic representation of APCI ion source.

Quadrupole mass analyzer

The mass analyzer is the heart of the mass spectrometer, which separates ions based on mass to charge ratios. There are several main types of mass analyzers including magnetic sector, time of flight, ion trap, and quadrupole which has been utilized in our studies. A quadrupole mass analyzer (Figure 10) consists of four parallel cylindrical rods that are arranged to a square. Each rod carries radio frequency (RF) and direct current (DC) voltage. The voltage applied to the quadrupole (RF/DC) determines which ions can reach the detector. Only ions with certain m/z ratios can pass the quadrupole. Due to their unstable trajectories, other ions, which collide with the quadrupole rods or are expelled from the quadrupole. The quadrupole not only functions as a mass selector allowing ions with specific m/z range to pass through but can also be operated in scan mode to conduct a spectrum scan by altering RF and DC range.

Triple quadrupole mass analyzer

As the name implies, a triple quadrupole consists of three quadrupoles namely Q1, Q2 and Q3 (Figure 11). Q1 and Q3 are working as mass filters, while Q2 is the collision cell to which a RF voltage is applied. Due to the introduction of neutral gas (often helium, nitrogen or argon), the pressure in Q2 is higher. The ions collide with neutral gas in the collision cell and is fragmented to product ions. The process in Q2 is called collision-induced dissociation (CID). The ions produced in Q2 through fragmentation are termed product ions. The ions generated in the ion source are called precursor or parent ions.

There are several typical scan modes that can be operated by a triple quadrupole: multiple reaction monitor (MRM) mode, product ion scan mode, precursor ion scan mode and neutral loss scan mode. In the MRM mode, Q1 and Q3 are set to select precursor ions and product ions, respectively. This mode is to quantify specific compounds in very high sensitivity (Lange et al., 2008).

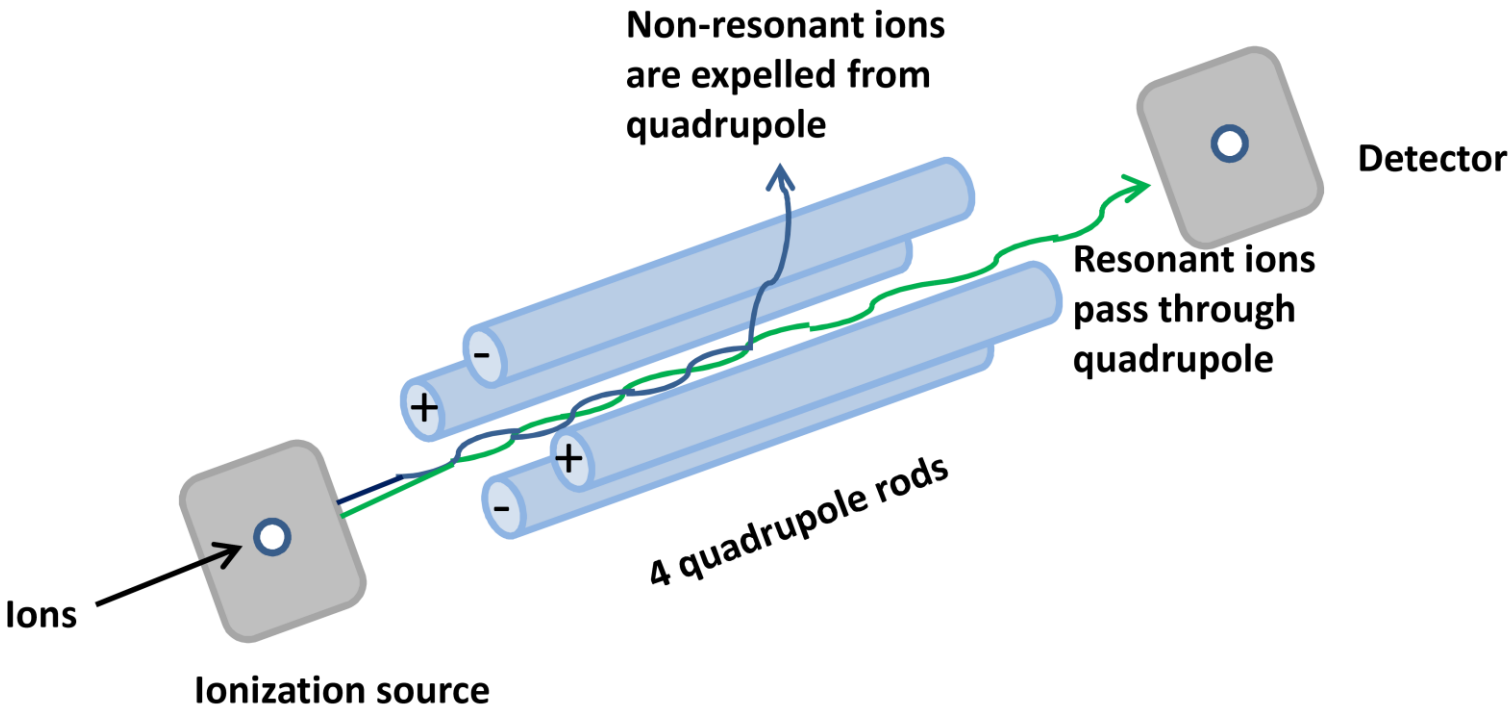


Figure 10. Schematic display of a quadrupole mass analyzer.

Detector

A key element of mass spectrometric systems is the detector. It is used to convert a current of individual ions into measurable signals. Some of the commonly used detectors in mass spectrometer are electron multipliers, array detector, photomultiplier conversion dynode and image current detector. In our work, the detector for the quadrupole mass spectrometer is an electron multiplier. An electron multiplier is a serial connection of dynodes which amplifies the current of separated ions into a measurable current of electrons. At the final dynode, the accumulated current is measured as a voltage pulse. In a mass spectrum, the X-axis stands for the m/z ratios and the Y-axis represents signal intensity.

1.2.1.3 LC-MS/MS

The combination of LC and MS was a significant achievement in the field of analytical chemistry. The liquid coming from an LC is inherently incompatible with a mass spectrometer operating under high vacuum (Ardrey, 2003). The ion source (e. g. ESI, APCI) of LC-MS/MS is able to transfer the sample molecules from liquid phase into the gas phase. The scheme of LC-MS/MS analysis in MRM mode is represented in Figure 11. A sample solution containing analtyes of interest are introduced to a LC column by mobile phases. The analytes with different physico-chemical properties are separated by the LC column. After elution from the LC column, the eluent is introduced to the mass spectrometer. The first component of an LC/MS/MS system is an ion source where the compounds are ionized and accelarated to a quadrupole mass analyzer. Only the ions with specific m/z ratios are selected to pass through the first quadrupole (Q1). The selected ions are then fragmented into product ions by collision with an inert gas in the collision cell (Q2). Generated product ions are transferred into the third quadrupole (Q3) where only ions with specific m/z ratios are allowed to pass.

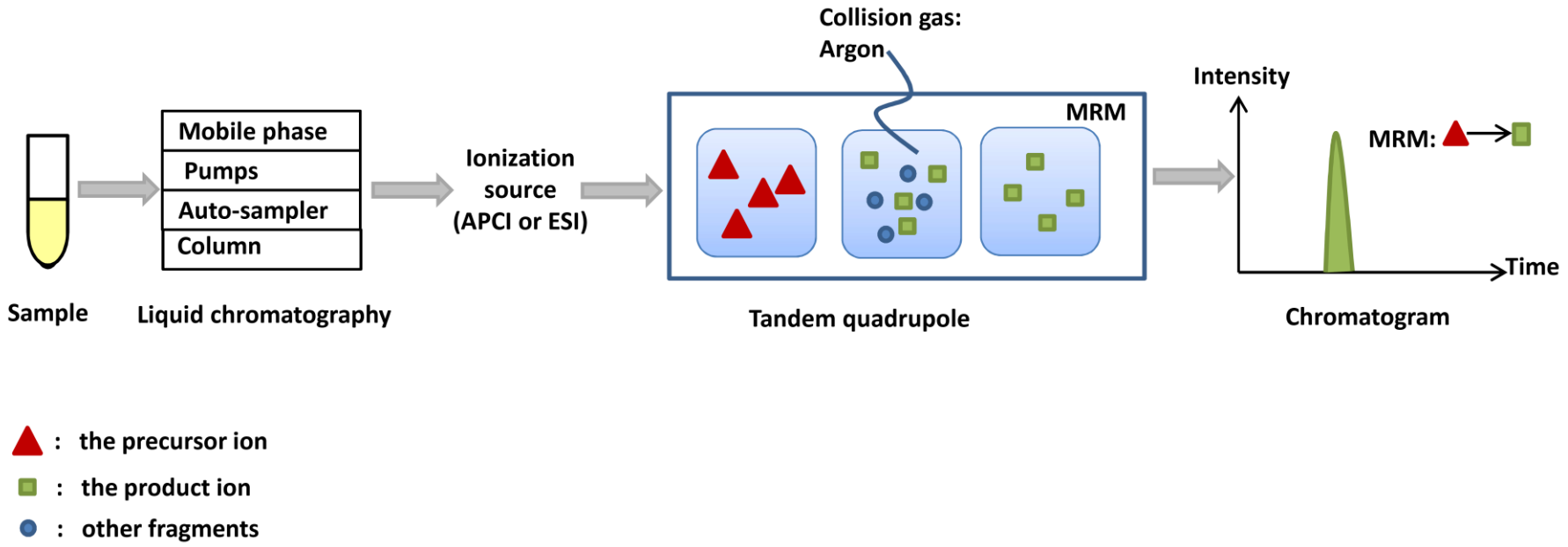


Figure 11. Representation of a typical LC-MS/MS operating in MRM mode. Samples are separated by liquid chromatography, ionized in the ion source, and enter the tandem quadrupole. Q1 selects the precursor ions, Q2 breaks the precursor ions into fragments and Q3 selects the product ions.

1.2.2 Gas chromatography-mass spectrometry (GC-MS)

1.2.2.1 Gas chromatography

Gas chromatography (GC) is another type of chromatography. GC is only applicable to volatile and thermally stable analytes (Sánchez et al., 2013). The mobile phase, usually an inert gas such as nitrogen or helium, carries the samples and passes through a stationary phase in the capillary column. The stationary phase is usually a film (e.g. dimethylpolysiloxane) coated on the inner wall of a capillary column and should be selected for the application. The capillary column is kept in an oven which can be programmed to ramp up/down the temperature. The compounds in the mixture can interact with the stationary phase at a different rate and show different affinities according to their physicochemical properties. Compounds with a weak affinity will be eluted first and the ones with a higher affinity will be retained on the column and elute later. The separation efficiecy can be affected by changing either the temperature of the stationary phase or the pressure of the mobile phase. As the temperature increases, compounds which have low boiling points elute from the column earlier than those which have higher boiling points. Therefore, there are two distinct separating forces, temperature and interactions between stationary phase and compounds. As the compounds are separated on the column, they elute from the column and enter a detector. The detector is able to creat an electronic signal whenever it detect the presence of a compound. The time used for each compound to reach the end of column is its retention time.

1.2.2.2 Mass spectrometry

Ion source of GC-MS

In contrast to LC-MS, in which ESI and APCI are employed, electron impact (EI) ionization, a hard ionization method, is typically employed in GC-MS. Here, the molecules in the gas phase enter the ion source and interact with energetic electrons to produce ions. The electrons, accelerated to 70 eV and concentrated in a beam, are emitted from a metal filament. Then the electron beam hits the sample gas to produce ions. The following diagram elucidates the mechanism of the EI ionization process (Figure 12).

The molecules are fragmented into ions of lower mass-to-charge ratios in a very reproducible and characteristic way. The method of fragmentation can be explained by

organic chemistry theory (Hamming, 1972). Therefore, the mass spectrum can be used to deduce structural information of the analyte. It is often applied to identify unknown compounds by comparing them with reference spectra from a library (Sánchez et al., 2013).

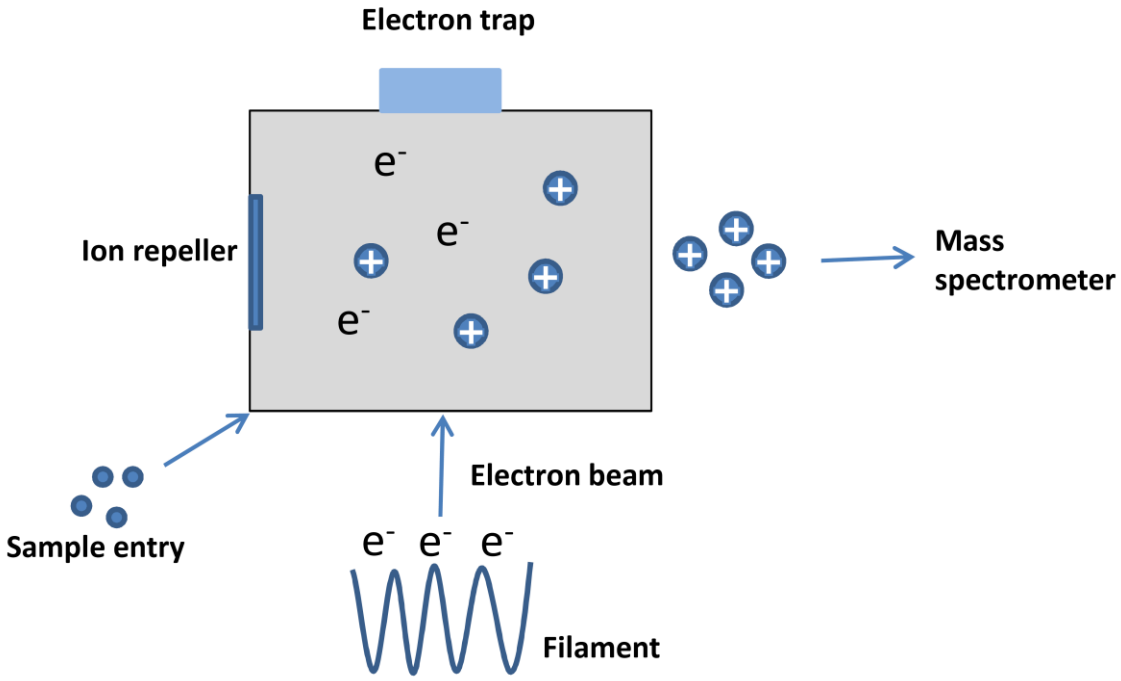


Figure 12. Diagram of an EI ion source. High-energy electrons are obtained from a heated filament and interact with the analytes to produce positive ions.

Quadrupole mass analyzer coupled to GC

The most common type of mass spectrometer (MS) hyphenated with a GC is the quadrupole mass spectrometer. The quadrupole mass analyzer can be operated in either a selective ion monitoring (SIM) mode or a full scan mode. Instead of measuring mass transitions as MRM mode, the mass analyzer is set to monitor the masses of interest in SIM mode. In SIM mode, only ion fragments with certain m/z ratios are selected to enter the quadrupole. In addition, the detection limit in SIM mode is lower than MRM mode since the quadrupole is only measuring a small number of fragments during each scan. More scans can take place each second, which enhances the sensitivity for predefined ions.

Detector of a typical GC-MS

The detector of GC-MS is an electron multiplier. it is a series of connected dynodes which can emit electrons when an ion with kinetic energy hits it. This process of emitting electrons is termed as "secondary emission". The electrons, increased gradually by repeating this process, are then detected, and displayed as a mass spectrum. In a mass spectrum, the X-axis is the m/z ratios, and the Y-axis is signal intensity.

1.2.2.3 GC-MS

GC-MS is the combination of GC and MS. It can be applied to analyze compounds both in a qualitative and quantitative way. In short summary, the vaporized samples are injected onto the column kept in a temperature-controlled chamber. The samples get separated on the column as the carrier gas moves through the column, and then enters the ion source. They are bombarded with a stream of energetic electrons, which can ionize and fragment the molecules. The formed ions include the molecular ions and/or ions from fragmentation. The ions are accelerated and rapidly selected according to their m/z ratios in a mass analyzer. The abundance of ions is then detected by measuring the current of generated electrons when the ions strike the detector. MS, serving as the detector for GC, generates a chromatogram indicating the quantity of compounds and their retention times (Figure 13).

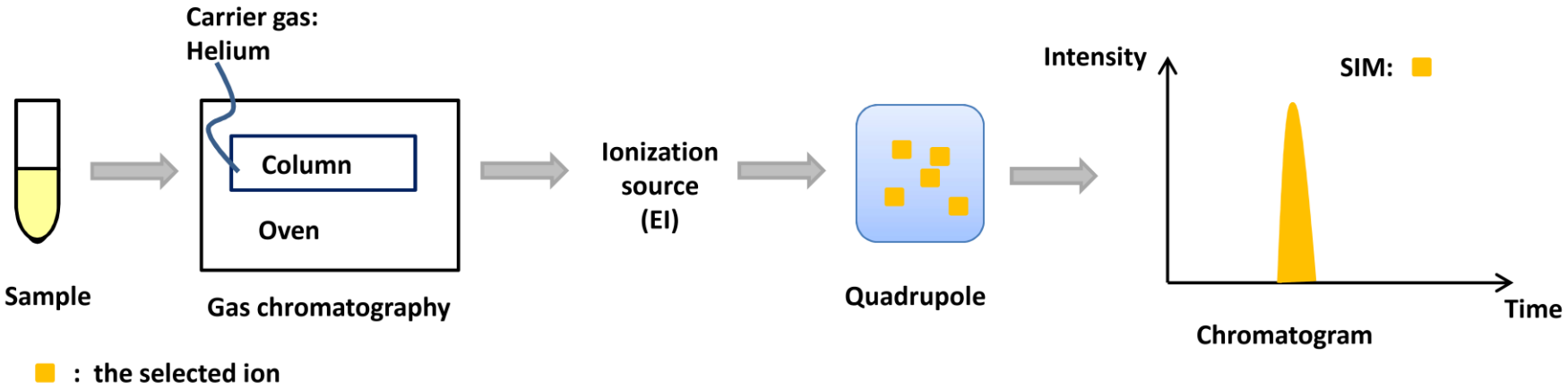


Figure 13. Representation of GC-MS analysis in SIM mode. Samples are separated by gas chromatography, ionized in the ion source, and enter the quadrupole. The selected ions are monitored and detected.

1.2.3 Steroid analysis by mass spectrometry

1.2.3.1 Steroid analysis by immunoassay vs. mass spectrometry

Reliable determination of steroids in biological fluids is essential for clinical diagnosis and research (Wudy et al., 2018). Steroids were measured traditionally by immunoassays, e.g. radioimmunoassay. At that time, immunoassays provided the opportunity to measure active steroid molecules directly in biological fluids. It needs less sample preparation and analyzing time is saved (Krasowski et al., 2014). However, cross reactivity between structurally similar steroids posed a challenge for the specificity of immunoassays (Taylor et al., 2015). Due to the complex components of biological samples, the matrix can affect the measurements when the samples are directly analyzed. Significant inter-laboratory variability was also observed even in commonly applied assays (Kalogera et al., 2013). Moreover, immunoassays cannot measure different analytes simultaneously because of the specific antibody and antigen reaction.

When we retrospectively reviewed the publications over the last few decades, we found an increase in the number of applications of mass spectrometry in steroid analysis. The fast development of mass spectrometry in steroid analysis is mainly because of its better specificity, high sensitivity and reproducibility (Shackleton, 2010). Steroidomics refers to the systematic study of as many steroids as possible in a given system. MS coupled with separation techniques such as LC or GC has the ability to analyze multiple steroids simultaneously. MS plays an increasingly important role in the studying steroidomics and diagnosing disorders of steroid synthesis and metabolism. There is little doubt that mass spectrometry methods will become the superior method in the future for steroid analysis (Taylor et al., 2015).

1.2.3.2 Steroid analysis by LC-MS/MS vs. GC-MS

The first application of the online chromatography-mass spectrometry method to steroid hormone analysis can be traced back to 1960s (Shackleton, 2010). GC-MS represents an advanced technique with high chromatographic resolution and reproducible ionization. GC-MS has the advantage of having the better chromatographic resolution and it is a superior technology for the separation of epimeric steroids. The hard ionization technique (ion impact ionization) applied in GC-MS can completely fragment the molecule ions

into different product ions in a repetitive way (Bloem et al., 2015). Furthermore, GC-MS in scan mode is considered as a discovery tool for novel steroid metabolites and is suitable for non-targeted steroid profiling. However, due to the necessary derivatization step and the long GC column, GC-MS analysis is time-consuming for sample workup and analysis.

The combination of quadrupole mass analyzer with HPLC were developed in 1980s. LC-MS, combining the separation abilities of HPLC together with the mass analysis capabilities of mass spectrometry, is a powerful tool in steroid. It is increasingly used to replace immunoassay methods because of its better sensitivity and specificity. LC-MS/MS is also the method of choice for the determination of intact conjugated steroids (Wudy & Choi et al., 2016). The soft ionization techniques (typically APCI or ESI) employed in LC-MS/MS make the quantification of intact steroid conjugates (steroid sulfates or glucuronides) feasible because steroid conjugates always show specific fragmentation patterns in triple quadruple mass spectrometry by losing the sulfated or glucuronidated group. Furthermore, the analysis time of LC-MS/MS is usually much shorter than GC-MS. Nowadays, LC-MS/MS is used for steroid analysis in clinical and research laboratory for its high throughput, good sensitivity, reduced sample preparation and complex steroid determination.

In conclusion, GC-MS and LC-MS/MS are two complementary techniques. LC-MS/MS is more suitable for targeted profiling of steroid metabolites and complex, e.g. intact conjugated steroids. GC-MS is excellent for characterizing “fingerprints” of steroids in a biological system.

Table 1. Comparison of the performance of LC-MS/MS and GC-MS in steroid analysis

Items	**LC-MS/MS**	**GC-MS**
sample preparation	less intensive	intensive
derivatization	less necessary	necessary
column	short	long
analysis time	short	long
resolution	poor	excellent
sample throughput	high	low
quantitative steroid analysis	yes	yes
intact steroid conjugates detection	yes	no
3-hydroxy-5-ene steroids quantification	poor	good
saturated steroids	poor	good
4-ene steroids	good	good
unknown steroids characterization	limited	good

1.2.3.3 Sample preparation in steroid analysis

Sample workup is a crucial step both for LC-MS/MS and GC-MS analysis. The complex compositions of biological samples can cause ion enhancement or ion suppression in ionization process. Moreover, the specificity of the measurement can be also affected. The ultimate objective of sample preparation is to extract a solution containing primarily analytes. Sample preparation should selectively remove interferences and extract compounds of interest from biological samples. In addition, some analytes are present in rather low concentrations in the samples, they can be also concentrated through sample workup. Therefore, very specific and effective sample workup procedures are needed.

Internal standards are commonly added before sample workup to correct for the loss of analytes during sample workup and analysis. The correct choice of internal standards in steroid analysis by LC-MS/MS and GC-MS can improve the accuracy and precision of analysis. The proper internal standards should be chemically similar to the analytes, but they should neither be naturally present in the samples nor reactive with the components in the samples. Internal standards should show the same behavior in terms of extraction, chromatography, and ionization as their corresponding analytes. The first choice of

internal standards would be corresponding stable isotopic labelled analytes. Structurally similar compounds e.g. epimers can be also used for internal standards if corresponding stable isotopic labelled internal standards are not available.

The most common methods used for sample cleanup are protein precipitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE). Proteins are generally present in biological fluids, such as serum, urine and amniotic fluid. They are large molecules which affect the measurements of steroids by blocking analytical columns due to their size. Also, they bind to steroids, which increases the irreproducibility of analysis, for example, the phenolic A-ring of estrogens can bind to proteins in serum (Sánchez et al., 2015). Protein precipitation is often done by adding organic solvents. Centrifugation is used to remove the precipitate in the samples. LLE separates compounds based on their solubility in two solvents with different polarities, usually water (polar) and organic solvents (non-polar). For SPE (Figure 14), a solid adsorbent is packed into a cartridge. As the sample passes through the cartridge, the compounds are retained by the stationary phase (e.g. silica gel). According to the physical and chemical properties of the compounds, the wash solvents are chosen to selectively remove interferences but retain analytes. Clean extracts are obtained by washing the cartridge with solvents which selectively elute the analytes. SPE is the method of choice in our studies (Wang et al., 2018) (Wang et al., 2021).

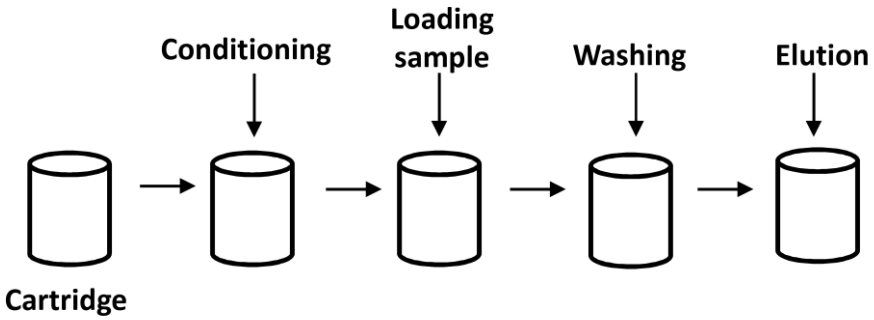


Figure 14. General steps for solid phase extraction

The sample preparation for GC-MS measurements, usually involving two steps, is different from that for LC-MS/MS. The first step is hydrolysis, which cleave the conjugate group from steroids. The second step is derivatization. Derivatization is usually not necessary in sample preparation for LC-MS/MS. Nevertheless, derivatization can also be applied to sample preparation for LC-MS/MS to improve the sensitivity of steroids.

GC-MS is suitable for the determination of volatile and thermally stable compounds. As steroids are not inherently volatile for GC-MS analysis, derivatization is necessary to increase their volatility and thermal stability of steroids. In our GC-MS study, we converted hydroxyl and ketone groups of steroids to methyloxime and trimethylsilyl derivatives, respectively (Wooding et al., 2013) (Figure 15). Derivatization modifies the physico-chemical properties of compounds enhancing their volatility and thermal stability and decreases the analytes' polarity. Moreover, derivatization increases molecular weight of analytes, separating them from low molecular weight interferences.

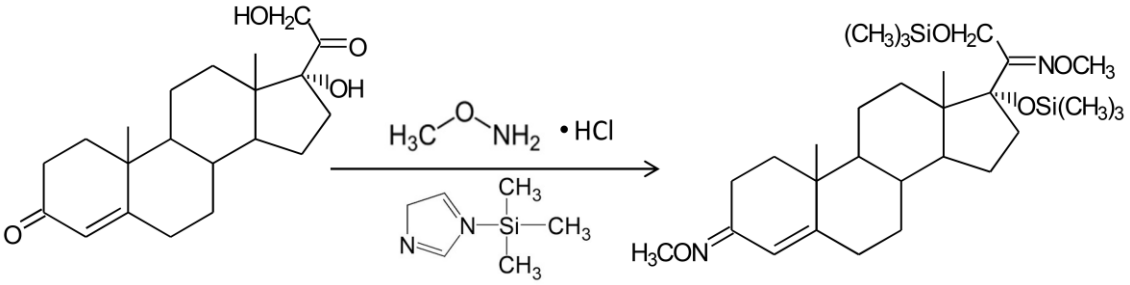


Figure 15. Methoximation and silylation of 17α-hydroxy progesterone, the keto groups are derivatized to methyloxime and the hydroxyl group is derivatized to trimethylsilyl ethers.

1.2.3.4 Method validation in steroid analysis

Method validation is of great importance in the establishment of an analytical method (Food and Drug Administration, 2018). A method can not be used for sample analysis before validation. The objective of method validation is to demonstrate the reliability of a method for determination of concentrations of analytes in a specific matrix. The method validation should be appropriate for the intended purpose of the study. The parameters of validation in our study should include accuracy, precision, lower limit of quantification (LLOQ), linearity of calibration curve, selectivity and matrix effect.

Accuracy of a method means the closeness of the measured concentration to the real concentration of an analyte. Accuracy is determined by analysis of quality controls containing known amounts of analytes. Accuracy should be measured using a minimum of 3 concentrations and 5 determinations per concentration. The mean values should be within 15% of the nominal values except at LLOQ, where it should not exceed 20%.

Precision describes the closeness of values obtained from replicate measurements of the same sample to each other. Precision should be measured using a minimum of 3 concentrations and 5 determinations per concentration. The precision should be determined as coefficient of variation (CV) at each concentration level. The values should not exceed 15% except for the LLOQ, where it should not exceed 20%.

One discriminates between intra-day accuracy/precision and inter-day accuracy/precision. Intra-day accuracy/precision assesses the accuracy/precision during a single analytical run. Inter-day accuracy/precision measures the accuracy/precision on five different runs (Wharf, 2011).

LLOQ is the lowest concentration of an analyte to be determined with acceptable precision and accuracy for a given method.

The calibration plot in bioanalytical method is the relationship between the concentration of the analyte and signal intensity. The two most common regression models used in LC-MS/MS and GC-MS are linear and quadratic regression models (Moosavi et al., 2018).

Selectivity is also called specificity. It is the ability of the method to differentiate and quantify the analyte of interest in a sample in the presence of other interference.

Matrix effect assessment evaluates the difference in response of ionization in the presence/absence of a matrix. It is investigated by plotting response ratios of spiked analytes in charcoal-treated fluids with full sample workup against the same concentrations of standards prepared directly in the reconstitution solution without sample workup. If there is an increase in response, it means the matrix enhances the ionization process (ion enhancement). Vice versa, a decrease of response means ion suppression.

1.3 Motivation and objective of the study

Steroids function biologically as signaling molecules and important components of cell membranes. They are present in biological fluids in unconjugated and conjugated forms, e.g. steroid sulfates and steroid glucuronides. To study the steroids in biological samples, appropriate analytical methods are required. Compared to immunoassay, LC-MS/MS and GC-MS are both advanced techniques in steroid analysis. LC-MS/MS with soft ionization such as APCI and ESI is suitable for the analysis of intact steroid conjugates. GC-MS which has the advantage of better chromatographic resolution is the superior technology to study metabolomics and metabolism disorders.

The objective of the study was to develop analytical methods and to determine the levels of steroids in various biological fluids based on mass spectrometry (LC-MS/MS and GC-MS).

The first objective was to develop and validate a sensitive and selective LC-MS/MS method for reliable determination of both free and sulfated steroids in human amniotic fluid (AF). The new LC-MS/MS method was then applied to establish reference values of 14 free and 6 sulfated steroids in AF of mid-gestation.

The second goal was to delineate steroid hormone metabolites in AF by a comprehensive targeted GC-MS method. The levels of 52 steroids including pregnenolone and 17-OH-pregnenolone metabolites, dehydroepiandrosterone and its metabolites, progesterone and 17-OH-progesterone metabolites, sex hormones as well as corticosterone and cortisol metabolites in AF of mid-gestation were measured.

A new analytical method was needed for future studies to investigate biological roles of steroid glucuronides. Thus, a new LC-MS/MS method for determining steroid glucuronides was developed and validated. The method was then used to gain a deeper understanding of steroid glucuronides excretion pattern in human urine.

2 Publications

Publication 1: **Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A LC-MS/MS study**.

Rong Wang, Dov Tiosano, Alberto Sánchez-Guijo, Michaela F. Hartmann, Stefan A. Wudy. Journal of Steroid Biochemistry and Molecular Biology. Volume 185, 2019, Pages 47-56. doi:10.1016/j.jsbmb.2018.07.007

Publication 2: **Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A GC-MS study**.

Rong Wang, Michaela F. Hartmann, Dov Tiosano, Stefan A. Wudy. Journal of Steroid Biochemistry and Molecular Biology. Volume 193, 2019, 105412. doi:10.1016/j.jsbmb.2019.105412

Publication 3: **Targeted LC-MS/MS analysis of steroid glucuronides in human urine**.

Rong Wang, Michaela F. Hartmann, Dov Tiosano, Stefan A. Wudy. Journal of Steroid Biochemistry and Molecular Biology. Volume 205, 2021, 105774. dio:10.1016/j.jsbmb.2020.105774

2.1 Publication 1

Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A LC-MS/MS study.

Rong Wang, Dov Tiosano, Alberto Sánchez-Guijo, Michaela F. Hartmann, Stefan A. Wudy. Journal of Steroid Biochemistry and Molecular Biology. Volume 185, 2019, Pages 47-56.

Abstract

Here we present a newly developed and fully validated LC-MS/MS method for the targeted analysis of 20 unconjugated and sulfated steroids in 65 amniotic fluid (AF) samples during mid-gestation. The method requires 600 µL of AF. Sample preparation included protein precipitation, centrifugation, solid phase extraction and derivatization. The method is capable of measuring progesterone, 17α-hydroxyprogesterone, testosterone, estrone, estradiol, estriol, estrone sulfate, 17β-estradiol 3-sulfate, estriol 3-sulfate (E3S), 17β-estradiol 17-sulfate, 16α-hydroxydehydroepiandrosterone sulfate (16OH-DHEAS), dehydroepiandrosterone sulfate (DHEAS), pregnenolone sulfate, 17α-hydroxypregnenolone sulfate, testosterone sulfate, epitestosterone sulfate, dihydrotestosterone sulfate, androsterone sulfate, epiandrosterone sulfate and 3,17β-androstenediol 3-sulfate. The performance of the method was evaluated at three quality control levels for each compound ranging from low limit of quantification to high limit of quantification. The method showed good accuracy and precision. Linearity for each analyte was above 0.99. The limit of quantification ranged between 0.2 and 1.7 ng/mL for the 20 compounds. Except DHEAS, all other sulfated steroids were quantified by LC-MS/MS in AF for the first time. We have produced MS based reference values for 20 steroids in AF of mid-gestation. DHEAS and E3S were both strongly correlated with 16OH-DHEAS, thus confirming the classic concept of the feto-placental unit. Our LC-MS/MS method can be used for future prenatal diagnosis of steroid metabolism disorders.

Contribution

The first author carried out the study according to the advice of her supervisor. The first author performed and optimized procedures for sample workup, instrumental analysis and data analysis. The first author created all the figures and tables for the manuscript and drafted the manuscript. The drafted manuscript was reviewed, supplemented and corrected together with all other co-authors.

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Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A LC–MS/MS study☆

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ABSTRACT

Growth and development of an embryo or fetus during human pregnancy mainly depend on intact hormone biosynthesis and metabolism in maternal amniotic fluid (AF). We investigated the hormonal milieu in AF and developed a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the determination of 14 sulfated and 6 unconjugated steroids in AF. 65 A F samples (male: female = 35: 30) of mid-gestation ranging from 16th week of gestation to 25th week of gestation were analyzed. Reference data of 20 steroid levels in AF of healthy women were provided. 13 sulfated and 3 unconjugated steroids were for the first time quantified in AF by LC–MS/MS. Highest concentrations were found for pregnenolone sulfate (PregS: mean ± SD, 8.6 ± 3.7 ng/mL), 17α-hydroxypregnenolone sulfate (17OHPregS: 4.9 ± 2.0 ng/mL), epitestosterone sulfate (eTS: 7.3 ± 3.6 ng/mL), 16α-hydroxydehydroepiandrosterone sulfate (16OH-DHEAS: 21.5 ± 10.7 ng/mL), androsterone sulfate (AnS: 9.2 ± 7.4 ng/mL), estrone sulfate (E1S: 3.0 ± 3.0 ng/mL), estriol 3-sulfate (E3S: 8.1 ± 4.0 ng/mL) and estriol (E3: 1.2 ± 0.4 ng/mL). Only testosterone (T) showed a significant sex difference ($p < 0.0001$). Correlations between AF steroids mirrored the steroid metabolism of the feto-placental unit, and not only confirmed the classical steroid pathway, but also pointed to a sulfated steroid pathway.

1. Introduction

Activity of the hypothalamus-pituitary-adrenal axis can be detected as early as 8th -12th week of gestation [1]. Corticotrophin releasing hormone (CRH) is produced by fetal hypothalamus and placenta. CRH stimulates the pituitary to secrete adrenocorticotrophic hormone (ACTH). ACTH stimulates fetal adrenals to synthesize dehydroepiandrosterone sulfate (DHEAS) from cholesterol. DHEAS from fetal adrenals is thereafter hydroxylated at position 16α in fetal liver by cytochrome P450 CYP3A7 [2]. DHEAS and 16OH-DHEAS are main precursors for estrogen production in placenta (Fig. 1).

Fetal plasma or serum directly reflects the hormone levels in fetuses. But it is highly risky and complicated to obtain such samples in utero. Some studies on steroid concentrations in umbilical cord blood were made after delivery [4]. AF which is swallowed and excreted by the fetus also provides information on fetal hormone concentrations [3].

Abbreviations: AF, amniotic fluid; LC–MS/MS, liquid chromatography-tandem mass spectrometry; CRH, corticotrophin releasing hormone; ACTH, adrenocorticotrophic hormone; DHEAS, dehydroepiandrosterone sulfate; 4A, 4-androstenedione; epiAn, epiandrosterone; CAH, congenital adrenal hyperplasia; MeOH, methanol; ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; SD, standard deviation; RIA, radioimmunoassay; DNSC, dansyl chloride; PregS, pregnenolone sulfate; 17OHPregS, 17α-hydroxypregnenolone sulfate; eTS, epitestosterone sulfate; eT, epitestosterone; 16OH-DHEAS, 16α-hydroxydehydroepiandrosterone sulfate; AnS, androsterone sulfate; E1S, estrone sulfate; E3S, estriol 3-sulfate; E3, estriol; Prog, progesterone; 17OHProg, 17α-hydroxyprogesterone; E1, estrone; E2, 17β-estradiol; E2S, 17β-estradiol 3-sulfate; TS, testosterone sulfate; DHTS, dihydrotestosterone sulfate; AnDiolS, 3β,17β-androstenediol 3-sulfate; d_3DHTS, [16,16,17α-D3] 5α-Androstan-17β-ol-3-one 17-sulfate; d_3TS, [16,16,17α-D3] 4-Androsten-17β-ol-3-one 17-sulfate; d_3eTS, [16,16,17β-D3] 4-Androsten-17α-ol-3-one 17-sulfate; d_9Prog, [2,2,4,6,6,17α,21,21,21-D9] 4-Pregnen-3,20-dione; $d_8$17OHProg, [2,2,4,6,6,21,21,21-D8] 4-Pregnen-17α-ol-3,20-dione; d_3T, [16,16,17-D3] 4-Androsten-17β-ol-3-one; d_4E2, [2,4,16,16-D4] Estra-1,3,5(10)-trien-317β-diol; d_3E3, [2,4,17-D3] 1,3,5(10)-Estratrien-316α,17β-triol; d_4E1S, [2,4,16,16-D4] Estra-13,5(10)-trien-3-ol-17-one 3-sulfate; d_4E2S, [2,4,16,16-D4] 17β-Estradiol 3-sulfate; d_6DHEAS, [2,2,3,4,4,6-D6] 5-Androsten-3β-ol-17-one 3-sulfate; d_4PregS, [17α,21,21,21-D4] 5-Pregnen-3β-ol-20-one 3-sulfate; $d_3$17OHPreg, [21,21,21-D3] 5-Pregnen-3β,17α-diol-20-one; $d_3$17OHPregS, [21,21,21-D3] 5-Pregnen-3β,17α-diol-20-one 3-sulfate

☆ This paper is dedicated to the 80th birthday of Prof. Janos Homoki, Ulm/Donau (Germany).

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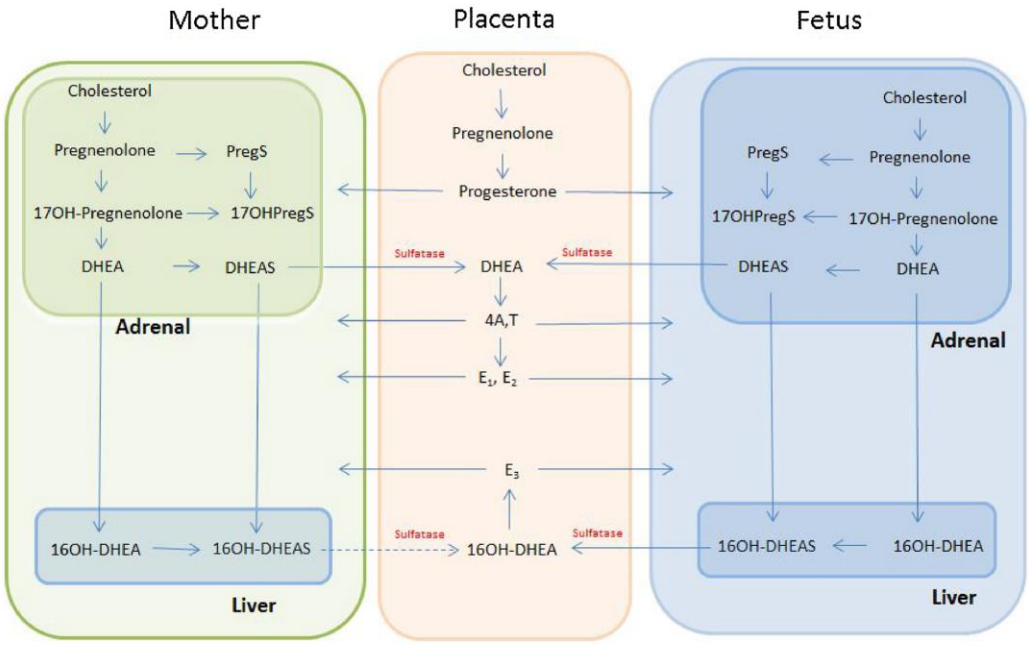

Fig. 1. Steroid metabolism of the feto-placental unit. 16OH-DHEAS from fetus contributes to 90% of placental E3 [3].

Studies found AF levels of testosterone and 4-androstenedione (4 A) to be higher in male than female fetuses during mid-gestation [5,6]. Furthermore, steroid analysis in AF allowed for the prenatal diagnosis of 21-hydroxylase deficiency [7,8].

Most studies on steroids in AF have been carried out by immunoassays for several decades [6,9–12]. Due to drawbacks of immunoassays such as cross-reactivity, less efficiency and matrix interferences, GC–MS and LC–MS nowadays have become the main prevalent tools for the qualitative and quantitative analysis of steroids [13]. Homoki et al. were the first to analyze AF samples by mass spectrometry [14]. Wudy et al. have been the first to publish stable isotope dilution/GC–MS based reference data on six unconjugated steroids in AF of mid-gestation of normal and congenital adrenal hyperplasia (CAH) fetuses [15]. Only one paper by Fahlbusch et al. has used LC–MS/ MS to analyze eleven AF steroids so far [16].

For a long time, the biological role of conjugated steroids was unclear. Only unconjugated steroids have been considered as active forms which pass through cell membrane to interact with nuclear receptors [17]. Recently, studies found that sulfated steroids have another biological function. They are not only water-soluble excretion products, but they can also enter cells by specific uptake carriers and modulate the intracellular milieu [18]. As far as we know, no other sulfated steroids, except for DHEAS, have yet been quantified in AF by LC–MS/MS. The current study provides reference data on multiple unconjugated and sulfated steroids via LC–MS/MS in AF.

2. Materials and methods

2.1. Chemicals

Progesterone (Prog), 17α-hydroxyprogesterone (17OHProg), estrone (E1), 17β-estradiol (E2) and estriol (E3) were obtained from Makor chemicals ltd. (Rehovot, Israel). Estrone sulfate (E1S), 17β-estradiol 3-sulfate (E2S), estriol 3-sulfate (E3S), 17β-estradiol 17-sulfate (E2-17S), 16α-hydroxydehydroepiandrosterone sulfate (16OH-DHEAS), dehydroepiandrosterone sulfate (DHEAS), pregnenolone sulfate (PregS), 17α-hydroxypregnenolone sulfate (17OHPregS), testosterone sulfate (TS), epitestosterone sulfate (eTS), dihydrotestosterone sulfate (DHTS), androsterone sulfate (AnS), epiandrosterone sulfate (epiAnS) and 3β,17β-androstenediol 3-sulfate (AnDiolS) were bought from Steraloids Inc. (Newport, RI). T was obtained from Sigma-Aldrich (Taufkirchen, Germany). [16,16,17α-D3] 5α-Androstan-17β-ol-3-one 17-sulfate (d_3DHTS), [16,16,17α-D3] 4-Androsten-17β-ol-3-one 17-sulfate (d_3TS) and [16,16,17β-D3] 4-Androsten-17α-ol-3-one 17-sulfate (d_3eTS) were from LGC Standards GmbH (Wesel, Germany). [2,2,4,6,6,17α,21,21,21-D9] 4-Pregnen-3,20-dione (d_9Prog), [2,2,4,6,6,21,21,21-D8] 4-Pregnen-17α-ol-3,20-dione ($d_8$17OHProg), [16,16,17-D3] 4-Androsten-17β-ol-3-one (d_3T), [2,4,16,16-D4] Estra-1,3,5(10)-trien-3,17β-diol (d_4E2), [2,4,17-D3]1,3,5(10)-Estratrien-3,16α,17β-triol (d_3E3), [2,4,16,16-D4] Estra-1,3,5(10)-trien-3-ol-17-one 3-sulfate (d_4E1S), [2,4,16,16-D4] 17β-Estradiol 3-sulfate (d_4E2S), [2,2,3,4,4,6-D6] 5-Androsten-3β-ol-17-one 3-sulfate (d_6DHEAS), and [17α,21,21,21-D4] 5-Pregnen-3β-ol-20-one 3-sulfate (d_4PregS) were purchased from C/D/N Isotopes Inc. (Quebec, Canada). The IS for 17OHPregS, [21,21,21-D3] 5-Pregnen-3β,17α-diol-20-one 3-sulfate ($d_3$17OHPregS), was synthesized in-house from $d_3$17OHPreg (C/D/N Isotopes Inc.) [19].

Water (LC–MS grade), ammonium hydroxide and formic acid were purchased from Fluka (Taufkirchen, Germany). Methanol (MeOH), acetonitrile (ACN), acetone, n-hexane, and chloroform were obtained from Merck (Darmstadt, Germany). Ammonium acetate, dansyl chloride and sulfur trioxide trimethylamine were from Sigma-Aldrich. SepPak C18 cartridges (200 mg) were from Waters Corporation (Milford, MA), Sodium bicarbonate and zinc sulfate were from Roth (Karlsruhe, Germany).

2.2. Sample collecting

65 pregnant women (21–43 years of age), which underwent

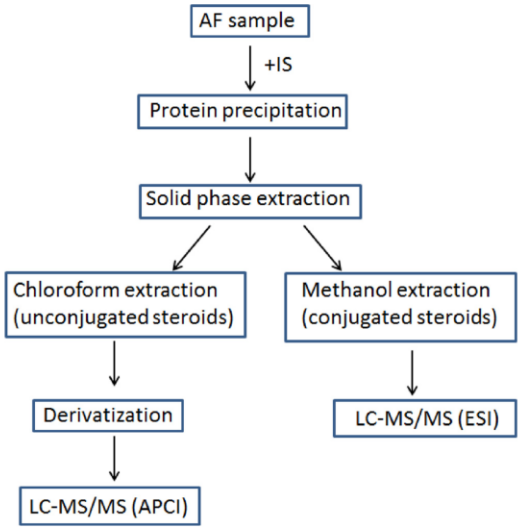

Fig. 2. Workflow of sample preparation.

amniocentesis agreed to participate in the study by allowing to investigate the amniotic fluids that remained after cytogenetic studies that were found to be normal. None of the pregnant women received any drug along the pregnancy. All participants signed a written informed consent and the study was approved by the Rambam Health Care Campus Helsinki Committee.

2.3. Sample preparation and analysis

The workflow of sample preparation is depicted in Fig. 2. 600 µL of AF were spiked with 25 µL of an internal standard cocktail, respectively. The concentration of each internal standard was 250 ng/mL. Samples were placed on the shaker for 15 min of mixing. Then the samples were precipitated by 1 mL of a solution consisting of 80% ACN and 20% aqueous $ZnSO_4$ (89 g/L) and shaken for 15 min After 10 min of centrifugation at 14,500 *g*, the supernatant was transferred to a glass tube with 3 mL of water and then mixed. Then a SepPak C18 cartridge was pre-conditioned with 3 mL of methanol and 3 mL of water. Thereafter the mixture was loaded onto the cartridge, washed first with 3 mL of water, then 3 mL of hexane. Then, with 4 mL of chloroform, the unconjugated steroids were eluted. After that, 4 mL of methanol were used to elute the sulfated steroids. The eluents from chloroform and methanol were both evaporated at 40 °C under a stream of nitrogen.

After chloroform extraction, a derivatization step was applied to improve the sensitivity for estrogens. The dried samples were cooled down to room temperature and then 100 µL of 0.1 M sodium bicarbonate solution was added to each sample, followed by addition of 100 µL of dansyl chloride acetone solution (1.0 mg/mL). Samples were capped, shaken well and put into a 60 °C heating block to react for 10 min. Fig. 3 shows the derivatization reaction. The derivatives were cooled down to room temperature again. Then the derivatives were evaporated, 200 µL of diluent (0.3% formic acid in 50% methanol/water) were added. An Agilent 1200SL HPLC system (Waldbronn, Germany) equipped with an Accucore defender guard column (10 × 2.6 mm, 10 µm, Thermo Fisher Scientific, Dreieich, Germany) and an Accucore Polar Premium column (50 × 4.6 mm, 2.6 µm, Thermo Fisher Scientific, Dreieich, Germany) was applied for chromatographic separation. LC–MS grade water served as mobile phase A, while methanol was used as mobile phase B. The injection volume was 30 µL. The detailed LC gradient can be found in **Supplementary data**. The total time for a single run was 8.5 min A Thermo Scientific triple quadrupole mass spectrometer (TSQ, Quantum Ultra, Dreieich, Germany) equipped with an APCI probe operating in positive mode was utilized for detection and quantification of steroids. The capillary temperature was set to 200 °C, and the vaporizer temperature was 350 °C. The sheath gas and arbitrary gas were 35 psi and 5 psi, the discharge current was 4.0, and the collision energy was 1.5.

The dried samples from the methanolic fraction were directly reconstituted with 200 µL of diluent (79.75% water, 10% MeOH, 10% ACN, and 0.25% ammonium hydroxide) respectively. Centrifugation was applied to remove undissolved substances. 20 µL of the final solution were injected on an Accucore Phenyl-X column (100 × 2.1 mm, 2.6 µm, Thermo Fisher Scientific, Dreieich, Germany) to achieve chromatographic separation. The solvent A was H_2O/ACN (85/15, v/v) with pH 7 adjusted by 10 mM ammonium acetate. Solvent B was MeOH/ACN (70:30, v/v). **Supplementary data** shows the detailed LC gradient. Electrospray ionization operating in negative mode was applied to analyze the samples. The capillary temperature was set to 270 °C, and the vaporizer temperature was 350 °C. The sheath gas was 50 psi. The auxiliary gas was 20 psi. The voltage was -3,500 V and the collision energy was 1.5.

2.4. Method validation

Linearity of the method was assessed by spiking different concentrations of standards and constant concentrations of IS into 600 µL of stripped urine for each analyte and was calculated by plotting response ratios of calibration points against their corresponding concentrations. The calibration range for each analyte is shown in Tables 1a and 1b. Limit of detection (LOD) was determined at a signal-to-noise ratio higher than 3. The lowest calibration level was considered as limit of quantification (LOQ). Quality controls (QCs) were prepared by spiking different concentrations of each analyte into stripped AF. Recovery of analytes was determined by comparing the response ratios of extracted QCs against that of spiked standards and IS in charcoal-treated AF post-extraction. The intra-day accuracy (%CV) and precision (%RE) of the method were evaluated by the results from five replicate analysis of QCs. The inter-day accuracy and precision were evaluated by analyzing 5 replicate QCs on each level on five different days. To evaluate urine as the surrogate matrix for AF, the calibration curve was prepared in stripped urine but QC samples were prepared in stripped urine and stripped AF. Acceptable accuracy for QCs prepared in both matrixes indicates the surrogate matrix is suitable.

2.5. Data analysis and statistics

Data analysis was performed by Microsoft excel 2010 and XLSTAT (https://www.xlstat.com/). Concentrations of steroids were shown as mean ± standard deviation (SD), median, minimum and maximum. The results of female fetuses and male fetuses were compared by Mann Whitney U test ($\alpha = 0.05$). Correlations were calculated by Pearson's correlation coefficient (R). The values close to −1 or 1 are strongly negatively or strongly positively correlated with each other [20].

3. Results

3.1. Sample information

The gestational age for samples was 18.8 ± 1.8 (Mean ± SD) weeks (median 18.3 weeks, range 16.0–24.6). The mothers' age was 35.6 ± 4.3 (Mean ± SD) years (median 36.5, range 21.0–43.0). No differences were found between males and females in terms of gestational weeks and mothers' age.

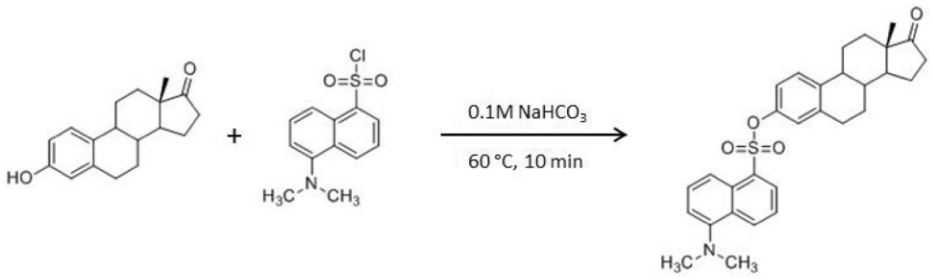

Fig. 3. The derivatization reaction between E1 and dansyl chloride was carried out at 60 °C for 10 min in 0.1 M $NaHCO_3$ buffer.

Table 1a
Validation parameters for unconjugated steroids in AF.

IS	Prog d_9Prog	17OHProg $d_8$17OHProg	T d_3T	E1 d_4E2	E2 d_4E2	E3 d_3E3
Linearity (ng/mL)	1.7-66.7	0.2-8.3	0.2-8.3	0.2-8.3	0.2-8.3	0.2-8.3
R^2	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
LOD (ng/mL)	0.3	0.1	0.1	0.1	0.1	0.1
LOQ (ng/mL)	1.7	0.2	0.2	0.2	0.2	0.2
LOQ Precision %CV	8.7	16.4	5.8	19.8	17.4	18.9
LOQ Accuracy %RE	117.9	105.9	104.5	88.2	95.8	113.6
Matrix effect						
QC 1	91.9	90.6	91.1	85.2	109.3	97.4
QC 2	98.3	102.5	101.5	111.0	90.4	110.4
QC 3	107.4	106.2	99.0	103.2	93.8	108.0
Intra-day precision (CV%)						
QC 1	1.9	8.2	4.2	12.7	11.0	14.1
QC 2	1.0	3.7	1.2	2.8	2.7	6.1
QC 3	1.4	3.6	0.7	5.6	1.8	3.2
Intra-day accuracy (CV%)						
QC 1	102.1	105.8	101.6	113.3	103.7	99.6
QC 2	104.8	98.6	102.9	101.7	102.0	98.8
QC 3	101.4	97.7	100.6	102.9	98.3	96.0
Inter-day precision (RE%)						
QC 1	7.3	5.3	8.9	12.4	3.8	13.1
QC 2	4.3	4.0	1.7	6.7	7.7	5.7
QC 3	4.6	10.0	1.4	6.6	9.8	4.4
Inter-day accuracy (RE%)						
QC 1	104.6	105.1	109.8	100.4	100.3	104.4
QC 2	102.1	98.1	102.6	104.4	94.0	106.6
QC 3	100.5	99.7	99.9	97.1	88.8	102.8
Recovery (%)						
QC 1	103.8	109.2	97.9	95.0	95.0	110.7
QC 2	109.9	113.0	93.5	107.7	90.5	107.3
QC 3	103.0	87.0	106.3	101.8	97.7	102.5
QCs (ng/mL)						
QC 1	2.5	0.25	0.25	0.25	0.25	0.25
QC 2	33.3	3.3	3.3	3.3	3.3	3.3
QC 3	66.7	6.7	6.7	6.7	6.7	6.7

3.2. Sample analysis and method validation

The chromatograms of sulfated steroids spiked into steroid-free matrix are shown in Fig. 4. Regarding the chromatograms of unconjugated and sulfated steroids of a real sample, refer to **Supplementary data.**

A concentration of derivatization buffer of 0.1 M sodium bicarbonate, 10 min of derivatization time, reconstitution solution (containing 0.3% HCOOH) were found to ensure optimal derivatization. We compared the chromatographic behavior of our analytes on two columns, Thermo Scientific Fisher C18 and Accucore Polar Premium. The Accucore Polar Premium column tended to be the optimal one for better resolution of analytes.

Mass spectrometry conditions were evaluated under different parameters including vaporizer temperature (450 °C, 400 °C, 350 °C), capillary temperature (325 °C, 275 °C, 200 °C), sheath gas (35 psi, 45 psi, 55 psi), auxiliary gas (2 psi, 5 psi, 10 psi) and discharge current (3.0, 4.0, 6.0). Best signals were obtained at 350 °C (vaporizer temperature), 200 °C (capillary temperature), 35 psi (sheath gas), 5 psi (auxiliary gas), and 4.0 (discharge current). MS transitions and parameters for sulfated steroids determination can be found in Reference [19].

The method validation followed the FDA (Food and Drug Administration, U.S. Department of Health and Human Services) and EMA (European Medicines Agency) guideline for bioanalytical method validation. The performance of the method is summarized in Tables 1a

Table 1b
Validation parameters for sulfated steroids in AF.

IS	PregS d_4PregS	17OHPregS $d_3$17OHPregS	16OH-DHEAS d_6DHEAS	DHEAS d_6DHEAS	AnDiolS d_6DHEAS	AnS d_3DHTS	epiAnS d_3DHTS	DHTS d_3DHTS	TS d_3TS	eTS d_3eTS	E1S d_4E1S	E2S d_4E2S	E2-17S d_4E2S	E3S d_4E2S
Linearity (ng/mL)	0.7-83.3	1.7-83.3	1.7-100.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3	0.7-83.3
R^2	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
LOD (ng/mL)	0.2	0.2	0.8	0.2	0.3	0.5	0.5	0.5	0.2	0.2	0.2	0.2	0.3	0.2
LOQ (ng/mL)	0.7	1.7	1.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	1.7
LOQ Precision (CV%)	10.3	17.3	4.9	13.0	19.0	19.5	13.0	17.8	11.7	7.6	7.0	6.4	3.6	7.43
LOQ Accuracy (RE%)	108.7	80.3	93.71	99.8	111.1	87.5	86.0	110.8	109.3	108.3	94.6	90.0	108.1	84.6
Matrix effect														
QC 1	103.1	87.9	93.81	112.7	95.7	107.4	106.4	113.8	100.6	99.5	95.2	88.8	105.3	85.2
QC 2	104.7	88.2	108.3	97.3	99.0	97.7	94.5	100.4	97.8	95.0	96.3	99.2	104.7	97.6
QC 3	98.6	96.5	104.3	103.8	102.6	101.1	104.8	101.7	98.4	103.0	98.4	102.0	103.7	113.5
Intra-day precision (CV%)														
QC 1	3.1	13.4	3.0	10.4	14.2	14.1	7.4	7.2	8.9	3.7	3.4	2.1	5.5	4.6
QC 2	7.0	1.0	2.1	6.8	9.4	11.5	3.2	1.8	1.7	1.5	3.8	1.5	3.9	7.5
QC 3	6.1	4.9	1.4	8.0	8.5	9.3	3.2	2.4	3.0	2.7	4.9	1.6	2.5	3.8
Intra-day accuracy (CV%)														
QC 1	97.6	98.9	107.0	88.0	88.6	90.1	98.3	102.0	106.4	97.2	93.8	92.1	110.6	92.0
QC 2	95.9	106.2	88.6	89.1	92.1	91.1	100.4	100.0	99.4	96.3	101.1	100.2	104.1	94.6
QC 3	98.4	102.2	94.2	90.0	92.6	89.1	104.9	104.4	99.8	100.6	101.6	102.9	102.6	91.2
Inter-day precision (RE%)														
QC 1	5.7	7.4	13.0	7.3	10.5	8.6	1.4	5.0	3.8	3.8	5.5	6.6	5.1	10.6
QC 2	4.3	7.3	6.0	7.6	4.4	5.9	2.5	3.4	5.3	3.2	2.3	3.5	2.8	3.1
QC 3	3.1	5.2	7.1	6.5	4.8	5.9	2.8	2.3	2.0	2.2	3.5	1.6	2.1	5.3
Inter-day accuracy (RE%)														
QC 1	107.0	105.7	95.99	100.8	101.6	99.6	98.8	104.4	104.7	99.7	103.4	103.6	106.9	90.5
QC 2	103.6	98.0	105.7	101.4	99.6	99.9	101.3	102.2	107.7	101.3	102.1	104.4	105.4	86.6
QC 3	103.0	106.3	104.2	101.3	99.5	98.2	103.6	103.9	102.1	103.9	106.3	104.6	106.2	89.9
Recovery(%)														
QC 1	103.0	86.6	94.2	93.6	96.7	102.5	90.4	110.1	104.9	98.2	104.1	99.7	100.5	100.7
QC 2	101.3	96.19	94.2	94.5	97.8	95.1	100.5	106.8	105.0	97.1	101.9	103.2	105.3	104.1
QC 3	102.6	98.5	103.2	104.7	102.6	101.7	103.9	107.5	108.3	108.0	103.5	106.0	110.4	112.7
QCs (ng/mL)														
QC 1	1.7	3.3	3.3	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	3.3
QC 2	8.3	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7
QC 3	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7	66.7

and 1b. The method showed good linearity in the calibration range, the linear regression model fitted all calibration curves well with $R > 0.99$ and the weighting factor 1/x for all analytes. As it can be seen in Tables 1a and 1b, the method showed good accuracy (unconjugated steroids: 88.8%–109.8%; sulfated steroids: 86.6%–110.6%) and precision (unconjugated steroids: 1.0%–14.1%; sulfated steroids: 1.0%–14.2%) for all QCs. Recovery was good for all QCs, ranging from 87.0% to 110.7% (unconjugated steroids) and from 86.6% to 112.7%. No matrix effects were observed for all QCs (unconjugated steroids: 85.2%–110.4%; sulfated steroids: 85.2%–113.8%).

3.3. Concentrations of steroids in AF

The hormone levels in AF were summarized in Table 2. The levels of Prog, 17OHProg, E3, 16OH-DHEAS, DHEAS, E3S, E1S, eTS, PregS, 17OHPregS and AnS in the samples lay within the calibration range. For T, E1, E2, AnDiolS, epiAnS, DHTS, TS, E2S and E2-17S, the concentrations were mostly below their LOQs. All the sulfated steroids we measured did not show any gender difference. Among the unconjugated ones, only T exhibited a significant sex difference ($P < 0.0001$).

3.4. Correlation of steroids

Correlation analyses were made between steroid concentrations of samples. Results were shown in Fig. 5. Strong positive correlations were found between 16OH-DHEAS and DHEAS, 16OH-DHEAS and E3S. Also, there were strong correlations between17OHPregS and PregS, E3 and E3S.

4. Discussion

4.1. Sample analysis and method validation

Protein precipitation and solid phase extraction are common sample preparation methods. They were used to obtain clean extracts of target analytes [27]. Sample preparation in our study was a modification of previous methods developed in our laboratory [19,28]: unconjugated steroids were eluted from SepPak C18 column by chloroform, sulfated steroids were eluted by methanol.

Derivatization was introduced to improve the sensitivity of estrogens. Dansyl chloride has been used as a derivatization agent in the determination of estrogens [29]. Sodium bicarbonate provided an alkaline environment. While the pH-values of 0.1 M and 0.2 M sodium bicarbonate solutions were both in the range of 8.0–9.0, respectively, we chose a 0.1 M $NaHCO_3$ because less acidic reconstitution solution was needed later for neutralization to facilitate ion detection in positive mode. The E1 and the E2 derivatives only differed by 2 Da (m/z 504.2 and 506.1), while they formed the same product ion. The product ion m/z 171.0 was formed in all estrogen-dansyl derivatives by cleavage of the S−C bond of the sulfonyl group. Therefore, baseline separation was needed. We chose an Accucore Polar Premium column because – in contrast to a C18 column – it permitted complete separation of all estrogen derivatives.

As not enough AF was available for method development and method validation, we had to find a suitable alternative. AF is a fluid similar to urine. It is swallowed and excreted via the kidneys by the fetus [30]. Human urine is affordable, easy to obtain, and identical to

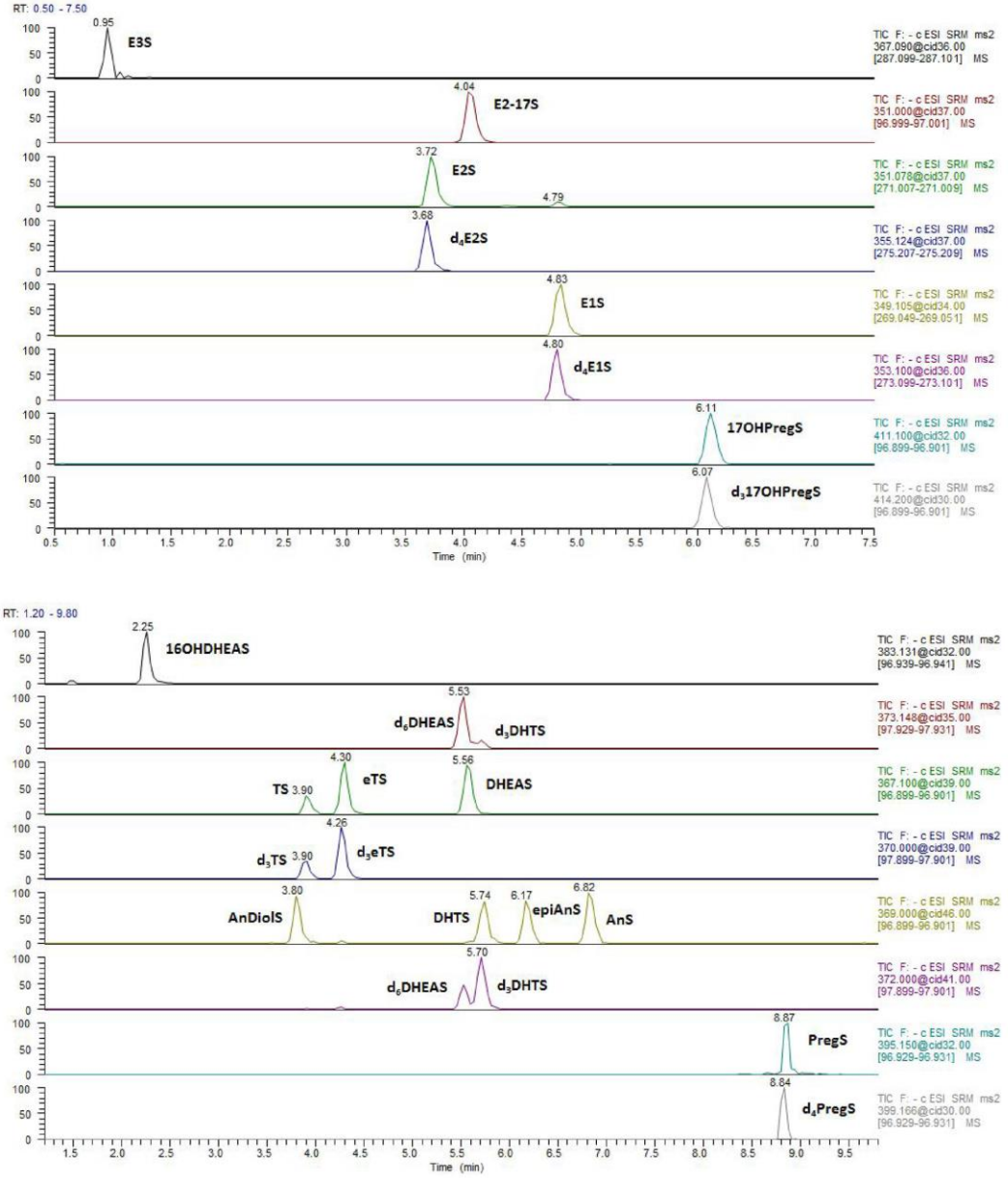

Fig. 4. Chromatograms of spiked standards sulfated steroids (10 ng/mL) in steroid-free matrix.

AF in terms of solubility and extractability [31]. To prepare stripped matrix, AF and urine were treated by charcoal. The evaluation of matrix effects showed that urine was adequate as a surrogate matrix for AF (see Tables 1a and 1b). Therefore, we chose urine as the surrogate matrix in our analysis.

4.2. The levels of steroids in AF of mid-gestation

Our results were compared with the available literature as shown in Table 2. The determination of steroids in AF in the last century was dominated by immunoassay. The first attempt to use mass spectrometry in determination of AF steroids was made by Homoki et al. [14]. In this study, AF steroids were measured after LH-20 fraction and enzymatic hydrolysis in pooled AF by gas chromatography followed by mass spectrometric identification (full scan modus). So far, most unconjugated steroids and DHEAS have been quantified by LC–MS/MS in AF. In this paper, we not only publish concentrations of various unconjugated, but also for the first time various sulfated steroids of AF in

Table 2
Concentrations of steroids (ng/mL) in AF during mid-gestation (13–28 weeks).

Steroids	Our data	Gender	Data from Literature				
	LC–MS/MS (n = 65)		LC–MS/MS [16]	GC/GC–MS	Immunoassay		
C 21 steroids							
Prog	37.4 ± 12.8, 33.9(16.4-78.6)	male	98.24 ± 63.76 85.75 (4.27-376.0)		46.39(18.41–116.90) [21]	55.0 ± 3.4 [22]	34.7 ± 4.1(7-97) [9]
		female	81.19 ± 52.86 66.85 (8.85-341.0)		47.21(21.23-104.37) [21]	54.0 ± 4.5 [22]	30.1 ± 4.0 (6-148) [9]
17OHProg	1.0 ± 0.3, 1.0(0.4-2.0)	male	1.81 ± 0.73 1.73 (0.67-3.64)		0.99 (0.75-1.34) [21]	1.62 ± 0.15(0.39-3.23) [9]	
		female	1.73 ± 0.87 1.59 (0.06-4.32)		1.21(0.98-1.49) [21]	1.51 ± 0.1(0.44-2.66) [9]	
		total		1.48(0.21-4.96) [15]			
PregS	8.6 ± 3.7, 8.2(2.6-20.3)	male			14.84 (8.97-24.55) [21]		
		female			17.06 (12.16-23.93) [21]		
		total					
17OHPregS	4.9 ± 2.0, 4.5(2.1-13.2)	total			2.0 ± 0.2 [23]		
C19 steroids							
T*	< LOQ -0.6	male	0.30 ± 0.15 0.28 (0.01-0.77)	0.24(0.00-0.50) [15]	0.23(0.14-0.37) [21]	0.224 ± 0.011 [6]	
	< LOQ-0.1	female	0.02 ± 0.02 0.01 (0-0.07)	0.00(0.00-0.27) [15]	0.049(0.034-0.072) [21]	0.039 ± 0.002 [6]	
TS	< LOQ -3.9	male			1.10 [5]		
		female			0.96 [5]		
eTS	7.3 ± 3.6, 5.9(2.9-17.8)	total					
DHEAS	4.6 ± 2.4, 3.8(1.5-12.3)	male	9.73 ± 8.61 7.68 (0.38-65.1)				
		female	8.67 ± 6.45 7.17 (0.38-43.3)				
		total		5.4(4.8-5.9) [22]	0.17 ± 0.09 [24]		
16OH-DHEAS	21.5 ± 10.7, 19.3(6.9-62.9)	total					
DHTS	< LOQ-7.0	total					
AnS	9.2 ± 7.4, 7.4(0.9-39.4)	total					
epiAnS	< LOQ -2.7	total					
AnDiolS	< LOQ -1.9	total					
C18 steroids							
E1	< LOQ -1.3	male			0.238(0.16-0.355) [21]	0.256 ± 0.018 [25]	0.353 ± 0.033 [6]
		female			0.244(0.16-0.368) [21]	0.303 ± 0.030 [25]	0.331 ± 0.028 [6]
E1S	< LOQ-25.3	total					
E2	< LOQ-0.5	male			0.042(0.016-0.11) [21]	0.327 ± 0.068 [10]	0.064 ± 0.004 [6]
		female			0.056 (0.035-0.09) [21]	0.321 ± 0.095 [10]	0.096 ± 0.008 [6]
E2S	< LOQ -2.3	total			3.1,1.4 [22]		
E2-17S	< LOQ -3.7	total					
E3	1.2 ± 0.4, 1.1(0.6-2.6)	male			1.271 ± 0.166 [10]		
		female			1.481 ± 0.131 [10]		
		total				4.79 ± 1.84 [24]	
E3S	8.1 ± 4.0, 6.5(2.2-21.0)	total			3.7-13.0 [26]#		

The results are expressed as Mean ± SD, Median (min-max). Sample quantities for reference: n = 351 [5]; n = 120 [6]; n = 76 [9]; n = 89 [10]; n = 77 [14]; n = 172 [15]; n = 63 [20]; n = 99 [21]; n = unkown [22]; n = 130 [23]; n = 99 [24].

* Means significant difference between genders ($P < 0.0001$).

Stands for the test method used was a fluorescence method.

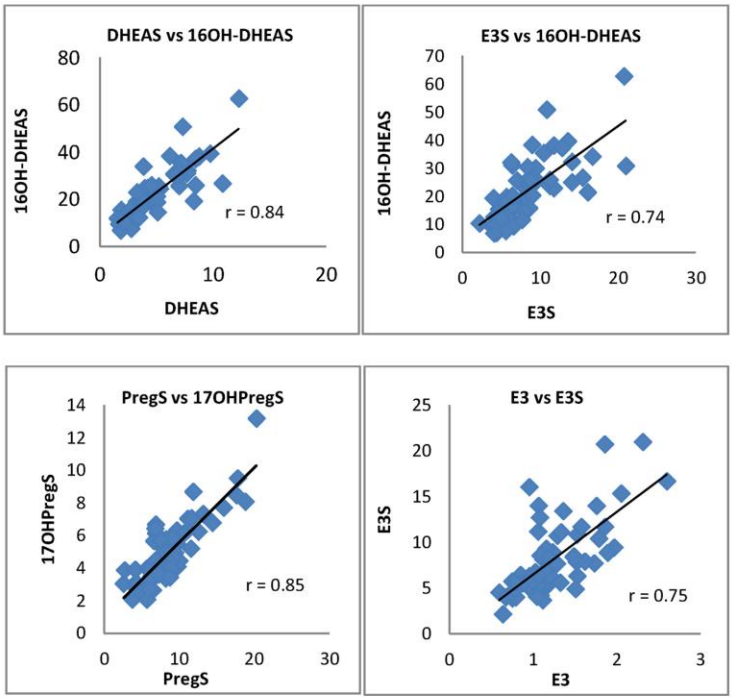

Fig. 5. Pearson's correlation between steroid concentrations in AF, X axis represents the concentration for one steroid (ng/mL) and Y axis means concentration for the other steroid (ng/mL). The correlation coefficient (r) between 0.7 and 0.9 means high positive correlation [20].

mid-pregnancy. The data from our study can be used as reference data for unconjugated and sulfated steroids in AF.

Concerning Prog, its mean concentrations were similar to data produced by various immunoassays [9,21,22]. Interestingly, our LC–MS/MS data were not consistent with those of another LC–MS/MS study [16],where the values scattered more extensively and mean values were 2–3 times higher. Our levels of 17OHProg were slightly lower than results from previous LC–MS/MS [16] and GC–MS studies [15]. Our values were rather close to those of Forest et al. [21] obtained by immunoassay, in which chromatographic purification had been used. Our mean concentrations of PregS were by 50% lower than those obtained by immunoassay [21]. This might be attributed to the lack of specificity of the immunoassay without extraction and chromatographic purification. 17OHPregS was higher than the value reported in 1978 by RIA [23], in which no corresponding internal standard for 17OHPregS was available.

The concentrations of T showed a significant sex difference. The difference was of comparable magnitude than has been found in other studies [15,16]. Epitestosterone (eT) and T are converted from 4 A by 17α-HSD and 17β-HSD, respectively. We found the levels of eTS to be approximately 6 times higher than the levels of TS. Interestingly, studies showed that the reaction from 4 A to T is reversible while converting 4 A to eT is irreversible [32]. DHEAS, the precursor especially for E1 and E2, was comparable with the value measured by GC as has already been described [22]. Whereas the values for DHEAS from other studies showed great variation (see Table 2). 16OH-DHEAS, the most important precursor for E3, showed the highest level of all sulfated steroids investigated. DHTS and AnDiolS were both low concentrated compounds. The concentrations of epiAnS were considerably lower than the concentrations of AnS. In most samples, epiAnS was below LOQ.

The concentrations of E1, E2, E2S and E2-17S were mostly below their LOQs. No literature was available for the levels of E1S, E2S and E2-17S in AF of mid-gestation. E3 was the dominating estrogen. Previous studies on the levels of E3 in AF were made mainly by immunoassay. First MS based data on the concentrations of E3 have now been provided. The E3 levels were similar to those obtained by Warne et al. [10] by radioimmunoassay (RIA). E3S showed the highest concentrations among all estrogens measured. Young et al. analyzed this compound in 7AF samples at mid-pregnancy by a fluorescence method, and found it to lie in a comparable range [26].

4.3. Steroids pathway during mid-pregnancy

Pearson's Correlation was used to study the linear relationship between individual concentrations of steroids and to look for interdependence between steroids. The correlations of steroid concentrations confirmed the classical steroid pathway and pointed to a sulfated steroid pathway in the feto-placental unit (Fig. 1) [33].

DHEAS correlated highly with 16OH-DHEAS. This finding supports the concept of the classical steroid pathway in the feto-placental unit [1]. The fetal adrenal produces large amounts of DHEAS. Then DHEAS is 16α-hydroxylated in the fetal liver to yield 16OH-DHEAS. Strong correlation was further found between 16OH-DHEAS and E3S. 16OH-DHEAS is the principal precursor of estriol. It is metabolized to E3 by cleavage of the sulfate group (placental sulfatase), conversion of the Δ5 double bond to a Δ4 double bond (3β-hydroxysteroid dehydrogenase), reduction of the 17-keto group (17β-hydroxysteroid dehydrogenase) and aromatization (aromatase). Then, E3 is sulfated at position C-3 by sulfotransferase to yield E3S [34]. DHEAS synthesized in fetal and maternal adrenals can also be desulfated in the placenta and converted to E1 and E2.

Furthermore, a very good correlation was found between PregS and 17OHPregS in AF. It has previously been reported that PregS serves as a substrate for biosynthesis of 17OHPregS in vitro and in the human male [35,36]. These authors proposed existence of a sulfated steroid pathway. Regarding our data, this is also likely to be the case in the feto-placental unit.

5. Conclusion

To conclude, we have developed and validated a LC–MS/MS method for targeted profiling of C21, C19 and C18 steroids in AF of mid-gestation. Reference data for 14 sulfated and 6 unconjugated steroids in AF of mid-gestation were provided. Most of these steroids have been quantified in AF for the first time by LC–MS/MS.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2018.07.007.

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2.2 Publication 2

Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A GC-MS study.

Rong Wang, Michaela F. Hartmann, Dov Tiosano, Stefan A. Wudy. Journal of Steroid Biochemistry and Molecular Biology. Volume 193, 2019, 105412.

Abstract

To complement our study of amniotic fluid by LC-MS/MS, gas chromatography-mass spectrometry was employed to characterize the steroidal context of amniotic fluid (n=65; male: female = 35: 30) of mid-gestation (median: 18.8th week, range: 16.0th – 24.6th week) by a comprehensive targeted steroid hormone metabolomics approach. The levels of 52 steroids including pregnenolone and 17-OH-pregnenolone metabolites, dehydroepiandrosterone and its metabolites, progesterone and 17-OH-progesterone metabolites, sex hormones as well as corticosterone and cortisol metabolites were measured. The dominating steroids were the group of pregnenolone and 17-OH-pregnenolone metabolites (mean ± SD: 138.0 ± 59.3 ng/mL), followed by the group of progesterone and 17-OH-progesterone metabolites (107.3 ± 44.3 ng/mL), and thereafter DHEA and its metabolites (97.1 ± 56.5 ng/mL). With respect to sex steroids, only testosterone showed a significantly higher value in male fetuses ($p<0.0001$) reflecting testicular endocrine activity. Of all estrogen metabolites, estriol showed by far the highest concentrations (33.2 ± 26.1 ng/mL). Interestingly, cortisol metabolites were clearly present (59.6 ± 13.6 ng/mL) though fetal *de novo* synthesis of cortisol is assumed to start from the 28th gestational week onwards. Our comprehensive characterization of the steroidal milieu in amniotic fluid of mid-gestation shows the presence of all relevant classes of steroid hormones. The steroidal milieu in amniotic fluid mirrors the steroidome of the feto-placental unit. Our set of basic data lays the foundation for further studies characterizing various diseases affecting steroid metabolism.

Contribution

The first author carried out the study according to the advice of her supervisor. The first author collected the data and performed data analysis. The first author created all the figures and tables for the manuscript and drafted the manuscript. The drafted manuscript was reviewed, supplemented and corrected together with all other co-authors.

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Characterizing the steroidal milieu in amniotic fluid of mid-gestation: A GC-MS study

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ABSTRACT

Intact steroid hormone biosynthesis is essential for growth and development of the human fetus and embryo. In the present study, gas chromatography-mass spectrometry was employed to characterize the steroidal milieu in amniotic fluid (n = 65; male: female = 35: 30) of mid-gestation (median: 18.8th week, range: 16.0th – 24.6th week) by a comprehensive targeted steroid hormone metabolomics approach. The levels of 52 steroids including pregnenolone and 17 – OH-pregnenolone metabolites, dehydroepiandrosterone (DHEA) and its metabolites, progesterone and 17 – OH-progesterone metabolites, sex hormones as well as corticosterone and cortisol metabolites were measured. The dominating steroids were the group of pregnenolone and 17 – OH-pregnenolone metabolites (mean ± SD: 138.0 ± 59.3 ng/mL), followed by the group of progesterone and 17 – OH-progesterone metabolites (107.3 ± 44.3 ng/mL), and thereafter DHEA and its metabolites (97.1 ± 56.5 ng/mL). With respect to sex steroids, only testosterone showed a significantly higher value in male fetuses ($p < 0.0001$). Of all estrogen metabolites, estriol showed by far the highest concentrations (33.2 ± 26.1 ng/mL). Interestingly, cortisol metabolites were clearly present (59.6 ± 13.6 ng/mL) though fetal *de novo* synthesis of cortisol is assumed to start from gestational 28th week onwards. Our comprehensive characterization of the steroidal milieu in amniotic fluid of mid-gestation shows presence of all relevant classes of steroid hormones and provides

Abbreviations: AF, amniotic fluid; LC–MS/MS, liquid chromatography-tandem mass spectrometry; GC–MS, gas chromatography-mass spectrometry; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; PregS, pregnenolone sulfate; 17OHPregS, 17α-hydroxypregnenolone sulfate; eTS, epitestosterone sulfate; DHEAS, dehydroepiandrosterone sulfate; 16OH-DHEAS, 16α-hydroxy-dehydroepiandrosterone sulfate; epiAnS, epiandrosterone sulfate; AnS, androsterone sulfate; E1S, estrone sulfate; E3S, estriol 3-sulfate; E3, estriol; Prog, progesterone; 17OHProg, 17α-hydroxyprogesterone; E1, estrone; E2, 17β-estradiol; E2-17S, 17β-estradiol 17-sulfate; E2S, 17β-estradiol 3-sulfate; TS, testosterone sulfate; DHTS, dihydrotestosterone sulfate; AnDiolS, 3β,17β-androstenediol 3-sulfate; An, 5α-Androstane-3α-ol-17-one (Androsterone); Et, 5β-Androstane-3α-ol-17-one (Etiocholanolone); DHEA, 5-Androstene-3β-ol-17-one (Dehydroepiandrosterone); 16α – OH-DHEA, 5-Androstene-3β,16α-diol-17-one; A5-3β, 17α, 5-Androstene-3β,17α-diol; A5-3β, 17β, 5-Androstene-3β,17β-diol (Androstenediol-17β); A5T-16αα, 5-Androstene-3β,16α,17β-triol (Androstenetriol-16α); PD, 5β-Pregnane-3α,20α-diol (Pregnanediol); PT, 5β-Pregnane-3α,17α, 20α-triol (Pregnanetriol); P5D, 5-Pregnene-3β,20α-diol (Pregnenediol); P5T-17α, 5-Pregnene-3β,17α,20α-triol (Pregnenetriol-17α); Po-5β, 3α, 5β-Pregnane-3α,17α-diol-20-one (17α – OH-Pregnanolone); Po-5α, 3α, 5α-Pregnane-3α,17α-diol-20-one; F, 4-Pregnene-11β,17α,21-triol-3,20-dione (Cortisol); THE, 5β-Pregnane-3α,17α,21-triol-11,20-dione (TH-Cortisone); THF, 5β-Pregnane-3α,11β,17α,21-tetrol-20-on (TH-Cortisol); α-THF, 5α-Pregnane-3α,11β,17α,21-tetrol-20-one (Allo-TH-Cortisol); α-Cl, 5β-Pregnane-3α,17α,20α,21-tetrol-11-one (α-Cortolon); β-Cl, 5β-Pregnane-3α,17α,20β,21-tetrol-11-one (β-Cortolone); α-C, 5β-Pregnane-3α,11β,17α,20α,21-pentol (α-Cortol); β-C, 5β-Pregnane-3α,11β,17α,20β,21-pentol (β-Cortol); 6β – OH-F, 4-Pregnene-6β,11β,17α,21-tetrol-3,20-dione; 20α-DHF, 4-Pregnene-11β,17α,20α,21-tetrol-3-one; 11 – OH-An, 5α-Androstane-3α,11β-diol-17-one; 11-O-An, 5α-anedrostane-3α-ol-11,17-dione; 11 – OH-Et, 5β-androstane-3α,11β-diol-17-one; 11-O-PT, 5β-Pregnane-3α,17α,20α-triol-11-one; THA, 5β-Pregnane-3α,21-diol-11,20-dione; THB, 5β-Pregnane-3α,11β,21-triol-20-one (TH-corticosterone); α-THB, 5α-Pregnane-3α,11β,21-triol-20-one (Allo-TH-Corticosterone); THS, 5β-Pregnane-3α,17α,21-triol-20-one; 15β – OH-Po, 5β-Pregnan-3α,15β,17α-triol-20-one; 16β – OH-DHEA, 5-Androstene-3β,16β-diol-17-one; 15β, 16α – OH-DHEA, 5-Androstene-3β,15β,16α-triol-17-one; 16-O-A5D, 5-Androstene-3β,17β-diol-16-one; A5T-16β, 5-Androstene-3β,16β,17β-triol; 16α, 18 – OH-DHEA, 5-Androstene-3β,16α,18-triol-17-one; 5β, 17α – OH-P5olon, 5-Pregnene-3β,15β,17α-triol-20-one; 16α – OH-P5olon, 5-Pregnene-3β,16α-diol-20-one; P5-tetrol-15β, 5-Pregnene-3β,15β,17α,20α-tetrol; 21 – OH-P5olon, 5-Pregnene-3β,21-diol-20-one; 6α – OH-THE, 5β-Pregnane-3α,6α,17α,21-tetrol-11,20-dione; 1β-OH-THE, 5β-Pregnane-1β,3α,17α,21-tetrol-11,20-dione; P5-3β, 20α,21-triol, 5-Pregnene-3β,20α,21-triol; 6α-OH-α-Cl, 5β-Pregnane-3α,6α,17α,20α,21-pentol-11-one; 6α – OH-β-Cl, 5β-Pregnane-3α,6β,17α,20β,21-pentol-11-one; 1β – OH-β-Cl, 5β-Pregnane-1β,3α,17α,20β,21-pentol-11-one; P5-tetrol, 5-Pregnene-3β,16α,20α,21-tetrol

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reference data. We conclude that the steroidal milieu in amniotic fluid mirrors the steroidome of the feto-placental unit.

1. Introduction

Human amniotic fluid (AF) is important for maintenance of pregnancy and fetal development. Apart from many other constituents, e.g. water, electrolytes, protein, phospholipids, etc., it also contains a multitude of steroids. Cholesterol, progestogens, androgens, estrogens, mineralocorticoids and glucocorticoids play significant roles in the homeostatic regulation of the feto-placental unit during the whole period of pregnancy [1]. For instance, progesterone maintains pregnancy by reducing myometrial contractility, whereas androgen biosynthesis and metabolism are essential for male sexual development [2].

The human adrenal anlage differentiates into two distinct zones by the eighth week of gestation: the inner large fetal/transient zone and the outer small adult/permanent zone [3]. The adult zone remains unchanged during entire pregnancy, while the fetal zone increases in size and production of steroids. The fetal zone starts to degenerate after delivery and disappears a few months later, whereas the adult zone increases and transforms to the adult gland [4]. There is also a third zone between the fetal zone and adult zone, which is called transitional zone [5].

The synthesis and catabolism of human steroids is complex. However, it is getting even more complex in the feto-placental unit because of the existence of the adrenal fetal zone from the first trimester onwards (Fig. 1). The compartment of the placenta and that of the fetus are specialized to produce certain steroids, respectively. The placenta synthesizes cholesterol from acetate. Cholesterol is then metabolized to large amounts of pregnenolone and progesterone. Due to the lack of 17-hydroxylase/1720-lyase, the placenta is not able to produce androgens by itself. Progesterone is a typical placental product: the fetal adrenal cannot synthesize progesterone from pregnenolone since it lacks 3β-hydroxysteroid dehydrogenase (3β-HSD). However, 17α-hydroxylase/17–20 lyase of the fetus allows the synthesis of DHEA and DHEA sulfate (DHEAS). DHEAS is then converted into 16α-hydroxydehydroepiandrosterone sulfate (16α − OH-DHEAS) by 16α-hydroxylase in fetal liver. 90% of placental 16α − OH-DHEA stems from the fetus, whereas 10% are of maternal origin [6]. In the placenta, 16α − OH-DHEAS is metabolized by sulfatase to 16α − OH-DHEA which is then aromatized to E3. E3 is thus an indicator of feto-placental function. Above mentioned steroids are then metabolized to more polar metabolites to be excreted.

Cortisol is important to maintain intrauterine homeostasis [7]. The transitional zone of the fetal adrenal is believed to be the site of fetal *de novo* cortisol production from the second half of gestation onwards. The transitional zone expresses 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) after the 28th week of gestation which allows the conversion of pregnenolone into 17α − OH-progesterone [8]. Thereafter, cortisol is synthesized through 21-hydroxylation and 11β-hydroxylation. Besides, maternal cortisol can also transfer across the placenta to reach out to the fetus. In addition to the above source, cortisol can be produced in the fetus from circulating placenta-derived progesterone [3].

Fetal zone steroids refer to those steroids which are only present in fetuses and newborns stemming from the fetal zone of the fetal adrenal, bearing a “3β-hydroxy-5-ene” structure. Most of these fetal zone steroids are hydroxylated in two or more positions, e.g. 1β, 6α/β, 15β, 16α/β and form sulfate conjugates [30]. Shackleton et al [9] were the first to identify 16β − OH-DHEA from the urine of 1–3 day-old infant. 5-Pregnene-3β,20α,21-triol (P5-3β,20α,21-triol) and 5-Pregnene-3β,16α,20α,21-tetrol (P5-tetrol) were identified from the infants of first six days of life by Shackleton [10]. Homoki et al [11] found fetal and neonatal “3β-hydroxy-5-ene” steroids e.g. 16α − OH-DHEA, 5-Pregnene-3β,16α-diol-20-one (16α − OH-P5olon) and related steroids, which were largely present in the 15th and 17th week of AF. They also identified two 15β-hydroxylated C_{21} steroids: 5-Pregnene-3β,15β,17α-triol-20-one (15β,17α − OH-P5olon) and 5-Pregnene-3β,15β,17α,20α-tetrol (P5-tetrol-15β).

In our last paper regarding AF, we characterized the levels of 6 unconjugated (Prog, 17OHProg, T, E1, E2 and E3) and 14 sulfated steroids (16OH-DHEAS, DHEAS, E3S, E1S, eTS, PregS, AnDiolS, epiAnS, DHTS, TS, E2S, E2-17S, 17OHPregS and AnS) in AF by LC–MS/MS [12]. To complement our findings of the LC–MS/MS study, we now used

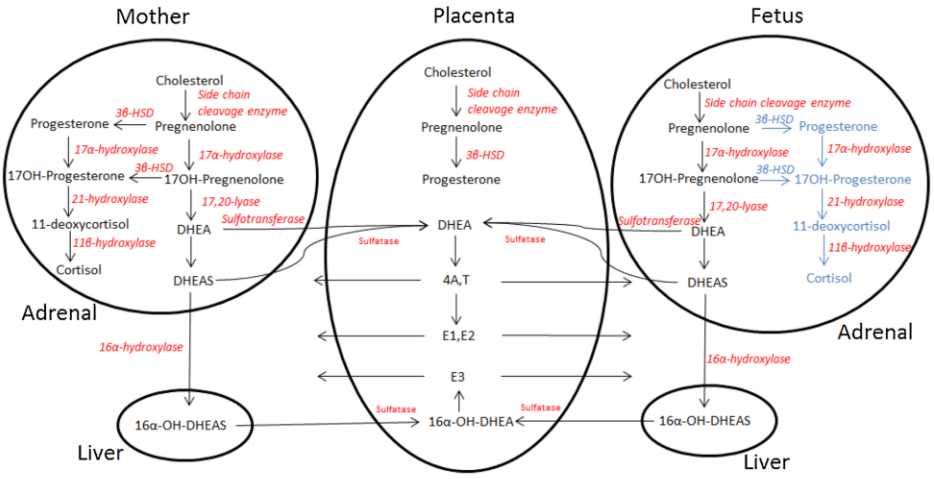

Fig. 1. Steroid metabolism and enzyme activities of the feto-placental unit. The enzymes and steroids shown in blue mark the pathway of *de novo* cortisol synthesis which takes place in the transitional zone of the fetal adrenal from the second half of gestation onwards. The figure was modified according to [2,12].

GC–MS to delineate further steroid hormone metabolites in AF of mid-gestation by a comprehensive targeted steroid hormone metabolomics analysis approach.

2. Materials and methods

2.1. Sample collection

65 pregnant women who underwent amniocentesis agreed to participate in the study. The gestational age of the fetuses was 18.8 ± 1.8 (Mean ± SD) weeks (range: 16.0–24.6). The mother's age was 35.6 ± 4.3 (Mean ± SD) years (range: 21.0–43.0). No sex difference was found in terms of gestational weeks and mothers' age. All participants signed a written informed consent and the study was approved by the Rambam Health Care Campus Helsinki Committee.

2.2. GC–MS targeted steroid metabolomics analysis

Steroids in AF samples were quantified by targeted GC–MS analysis by a modified protocol of our previous method [13]. In brief, free and conjugated steroids were extracted by solid phase extraction (Sep-Pak C18 cartridges; Waters, Milford, MA) from 5 ml of AF. The conjugates were enzymatically hydrolyzed (type H-1 sulfatase from helix pomatia; Sigma-Aldrich Chemie GmbH, Taufkirchen bei München, Germany), followed by recovery of the hydrolyzed steroids by a second solid phase extraction step. Known amounts of three internal standards (5α-androstane-3α,17α-diol, Paesel + Lorei, Frankfurt, Germany; stigmasterol and cholesteryl butyrate, Sigma-Aldrich Chemie, Steinheim, Germany) were added to a portion of each extract before formation of methyloxime-trimethylsilyl ethers. GC was conducted on an Optima-1 MS fused silica column (length 25 m, film thickness 0.1 μm, inner diameter 0.2 mm; Macherey-Nagel, Dueren, Germany) housed in an Agilent Technologies 6890 series GC equipped with an Agilent 7683 series injector that was directly interfaced to an Agilent Technologies 5975 inert XL mass selective detector (Agilent Technologies GmbH, Böblingen, Germany). Helium was used as carrier gas. The injections took place with the GC oven at 80 °C for 2 min. The temperature was gradually increased by 20 °C/min up to 190 °C (1 min). For separation of steroids, the temperature was increased by 2.5 °C/min to 272 °C. All steroids were analyzed using selected ion monitoring mode (analyte, *m/z*: P5D, 282.3; 21 – OH-P5olon, 490.4; 16α – OH-P5olon, 474.4; P5-3β,20α,21-triol, 267.2; P5-tetrol, 535.4; P5T-17α, 433.3; 15β,17α – OH-P5olon, 562.4; P5-tetrol-15β, 391.2; DHEA, 268.3; A5-3β,17α, 239.3; A5-3β,17β, 239.3; 16α – OH-DHEA, 446.4; 16β – OH-DHEA, 446.3; 16-O-A5D, 446.3; 16α,18 – OH-DHEA, 534.4; 15β,16α – OH-DHEA, 565.4; A5T-16β, 432.3; A5T-16α, 432.4; PD, 269.3; PT, 255.3; Po-5β,3α, 476.4; Po-5α,3α, 476.4; 15β – OH-Po, 564.5; 11-O-PT, 449.4; An, 270.3; Et, 270.3; 11 – OH-Et, 448.4; 11 – OH-An, 448.4; 11-O-An, 374.4; T, 389.4; E3, 504.4; E1, 371.3; E2, 416.4; THA, 400.3; α-THB, 564.4; THB, 564.4; THS, 564.4; F, 605.4; THF, 652.4; α-THF, 652.4; 6β – OH-F, 693.4; α-Cortol, 523.4; β-Cortol, 523.4; 20α-DHF, 296.3; THE, 578.5; α-Cortolone, 551.4; β-Cortolone, 449.3; 6α – OH-THE, 666.5; 6α – OH-β-Cl, 537.4; 6α – OH-α-Cl, 537.4; 1β – OH-THE, 666.4; 1β – OH-β-Cl, 562.4) [13,14,15,16].

2.3. Statistical analysis

Data analysis was made by Microsoft excel 2010 and OriginPro 2017 (64bit). Concentrations of steroids were shown as mean ± standard deviation (SD) as well as median and range (minimum - maximum). The concentrations measured below limit of detection (LOD) were treated as 0.0. The results of female fetuses and male fetuses were log transformed and compared by parametric comparison – Welch's two sample t-test. Since multiple comparisons were made, Bonferroni correction was conducted to protect from Type I Error. After adjustment, a p value < 0.001 was considered statistically significant.

Correlation analyses were made between steroid concentrations of samples. Correlations were calculated by Pearson's correlation coefficient (r) after log transformation of data. A p value $< 4 \times 10^{-5}$ was considered statistically significant after Bonferroni correction. The values close to -1 or 1 are intensively negatively or intensively positively correlated with each other.

3. Results

Fig. 2 shows the chromatograms of typical steroid metabolites in AF of mid-gestation. The levels of steroids measured were summarized in Tables 1a and 1b. In total, we analyzed the concentrations of 52 steroid hormone metabolites which included pregnenolone and 17 – OH-pregnenolone metabolites, DHEA and metabolites, progesterone and 17 – OH-progesterone metabolites, androgens, estrogens, corticosterone metabolites, 11-deoxycortisol metabolites, as well as cortisol and cortisone metabolites. Correlations between steroid hormone metabolites can be found in section on supplementary data.

The sum of pregnenolone and 17 – OH-pregnenolone metabolites showed the highest concentrations of all groups of steroids analyzed. In particular, P5-3β,20α,21-triol (mean ± SD: 45.3 ± 23.2 ng/mL) and

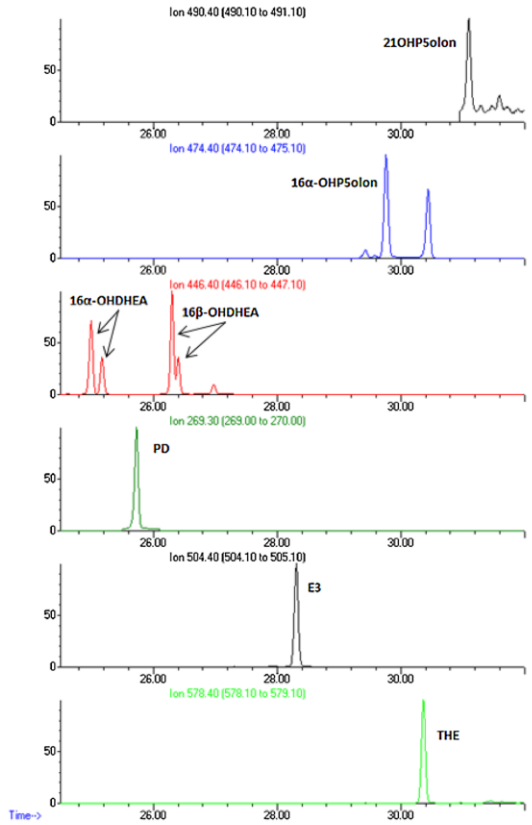

Fig. 2. Representative SIM-chromatograms of important steroids such as the pregnenolone metabolites 21 – OH-P5olon and 16α – OH-P5olon, the DHEA metabolites 16α – OH-DHEA and 16β – OH-DHEA (both appear as double peaks), the progesterone metabolite pregnanediol (PD), estriol (E3) and the cortisol metabolite tetrahydrocortisone (THE) in an AF sample of mid-gestation. (X: retention time, Y axis: abundance of signal.).

Table 1a
Concentrations of pregnenolone, progesterone, androgen and estrogen metabolites.

Steroids	Concentrations (ng/mL)			p value (male & female)
	Male	Female	Total	
Pregnenolone and 17-OH-pregnenolone metabolites (Δ5 unsaturated C_{21} steroids)				
P5D	0.5 ± 1.7	0.4 ± 1.2	0.5 ± 1.5	–
	0.0 (0.0-7.8)	0.0 (0.0-6.0)	0.0 (0.0-7.8)	
21-OH-P5olon	46.6 ± 24.0	39.3 ± 24.1	43.9 ± 24.0	0.42
	40.2 (0.0-122.6)	42.1 (0.0-123.5)	41.9 (0.0-123.5)	
16α-OH-P5olon	18.0 ± 7.6	16.4 ± 6.4	17.3 ± 7.1	0.34
	15.9 (9.1-50.8)	14.8 (9.7-45.1)	15.7 (9.1-50.8)	
P5-3β,20α,21-triol	48.2 ± 23.4	39.7 ± 22.9	45.3 ± 23.2	0.39
	44.0 (7.8-115.7)	39.1 (16.8-123.7)	41.5 (7.8-123.7)	
P5-tetrol	0.1 ± 0.2	0.0 ± 0.2	0.0 ± 0.2	0.08
	0.0 (0.0-1.2)	0.0 (0.0-0.9)	0.0 (0.0-1.2)	
Σ Pregnenolone metabolites	112.9 ± 46.1	95.4 ± 49.9	106.5 ± 48.0	0.18
	102.6 (31.3-243.8)	92.1 (32.0-292.3)	98.5 (31.3-292.3)	
P5T-17α	1.1 ± 0.5	2.9 ± 9.5	1.9 ± 6.4	0.91
	1.1 (0.0-2.4)	1.0 (0.5-52.9)	1.0 (0.0-52.9)	
15β,17α-OH-P5olon	16.5 ± 10.8	13.1 ± 10.8	15.7 ± 10.8	0.60
	14.3 (3.0-50.2)	13.2 (3.0-65.7)	14.0 (3.0-65.7)	
P5-tetrol-15β	15.8 ± 11.6	10.8 ± 6.8	13.8 ± 9.9	0.08
	12.8 (4.4-55.8)	10.3 (3.3-33.2)	11.2 (3.3-55.8)	
Σ 17-OH-pregnenolone metabolites	33.4 ± 20.4	26.7 ± 17.7	31.4 ± 19.1	0.34
	26.0 (9.6-107.2)	26.1 (8.3-100.4)	26.0 (8.3-107.2)	
Σ Pregnenolone and 17-OH-pregnenolone metabolites	146.3 ± 57.2	122.1 ± 61.3	138.0 ± 59.3	0.15
	137.6 (50.6-303.6)	114.9 (40.6-339.7)	133.4 (40.6-339.7)	
DHEA and metabolites (Δ5 unsaturated C_{19} steroids)				
DHEA	7.0 ± 3.4	8.6 ± 8.4	7.7 ± 6.2	0.38
	5.7 (3.1-16.3)	6.2 (3.1-50.6)	6.1 (3.1-50.6)	
A5-3β,17α	1.2 ± 0.4	0.9 ± 0.5	1.1 ± 0.5	0.03
	1.2 (0.0-2.1)	0.9 (0.0-2.3)	1.0 (0.0-2.3)	
A5-3β,17β	1.1 ± 0.5	0.9 ± 0.9	1.0 ± 0.7	0.69
	1.1 (0.0-1.9)	1.0 (0.0-4.2)	1.1 (0.0-4.2)	
16α-OH-DHEA	42.5 ± 22.4	42.1 ± 21.6	42.4 ± 21.8	0.92
	37.6 (16.3-133.2)	36.3 (22.5-133.4)	37.2 (16.3-133.4)	
16β-OH-DHEA	32.9 ± 29.9	32.8 ± 31.2	32.9 ± 30.3	0.92
	24.4 (7.4-185.1)	23.4 (12.6-182.1)	24.2 (7.4-185.1)	
16-O-A5D	6.4 ± 2.0	6.4 ± 1.6	6.4 ± 1.8	0.91
	6.0 (0.0-11.9)	6.1 (5.0-13.8)	6.0 (0.0-13.8)	
16α,18-OH-DHEA	3.7 ± 2.1	3.4 ± 2.4	3.6 ± 2.2	0.70
	3.1 (0.9-10.5)	2.6 (1.3-13.8)	3.0 (0.9-13.8)	
15β,16α-OH-DHEA	2.1 ± 1.5	1.6 ± 1.0	1.9 ± 1.3	0.28
	1.7 (0.0-7.3)	1.7 (0.5-6.2)	1.7 (0.0-7.3)	
A5T-16β	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.2	–
	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-1.2)	
A5T-16α	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–
	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	
Σ DHEA and metabolites	96.9 ± 56.2	96.7 ± 57.9	97.1 ± 56.5	0.95
	88.7 (38.6-356.7)	81.4 (49.0-361.5)	83.6 (38.6-361.5)	
Progesterone and 17-OH-progesterone metabolites				
PD	93.7 ± 36.0	87.8 ± 46.6	91.6 ± 41.0	0.44
	88.4 (33.9-195.8)	85.7 (37.9-279.9)	86.8 (33.9-279.9)	
PT	8.3 ± 3.6	9.7 ± 6.7	9.0 ± 5.3	0.32
	7.4 (3.7-17.9)	7.5 (4.4-33.0)	7.4 (3.7-33.0)	
Po-5β,3α	4.0 ± 2.0	4.8 ± 3.7	4.4 ± 2.9	0.35
	3.3 (2.0-9.4)	3.5 (2.4-18.3)	3.4 (2.0-18.3)	
Po-5α,3α	0.7 ± 0.3	1.0 ± 2.1	0.8 ± 1.5	0.92
	0.6 (0.4-1.4)	0.6 (0.4-12.3)	0.6 (0.4-12.3)	
15β-OH-Po	1.3 ± 0.5	1.3 ± 0.4	1.3 ± 0.5	0.66
	1.2 (0.5-3.0)	1.2 (0.6-2.2)	1.2 (0.5-3.0)	
11-O-PT	0.2 ± 0.3	0.3 ± 0.8	0.2 ± 0.6	0.99
	0.1 (0.0-0.9)	0.1 (0.0-4.5)	0.1 (0.0-4.5)	
Σ Progesterone and 17-OH-progesterone metabolites	108.2 ± 39.2	104.9 ± 50.2	107.3 ± 44.3	0.65
	98.4 (43.0-216.9)	102.5 (48.2-299.3)	101.6 (43.0-299.3)	
Other androgens (saturated C_{19} steroids)				
An	4.7 ± 1.7	4.4 ± 2.6	4.6 ± 2.1	0.68
	4.5 (1.9-9.3)	4.0 (0.0-12.1)	4.4 (0.0-12.1)	
Et	3.2 ± 1.4	4.0 ± 6.7	3.6 ± 4.6	0.87
	2.9 (1.5-6.6)	2.8 (1.2-38.9)	2.8 (1.2-38.9)	
11-OH-Et	1.0 ± 0.8	1.1 ± 0.9	1.1 ± 0.9	0.30
	1.0 (0.0-3.3)	1.1 (0.0-4.4)	1.1 (0.0-4.4)	
11-OH-An	1.0 ± 0.5	1.0 ± 0.8	1.0 ± 0.6	0.39
	0.9 (0.5-2.8)	0.9 (0.4-5.0)	0.9 (0.4-5.0)	

(*continued on next page*)

Table 1a (*continued*)

Steroids	Concentrations (ng/mL)			p value (male & female)
	Male	Female	Total	
11-O-An	0.7 ± 0.4 0.8 (0.0-1.3)	0.7 ± 0.4 0.8 (0.0-1.4)	0.7 ± 0.4 0.8 (0.0-1.4)	0.85
T	1.5 ± 0.4 1.5 (0.9-2.6)	1.1 ± 0.2 1.1 (0.7-1.5)	1.3 ± 0.4 1.2 (0.7-2.6)	< 0.0001*
Estrogens (C_{18} steroids)				
E3	36.8 ± 33.7 26.4 (10.8-188.0)	28.4 ± 11.6 25.4 (13.0-62.1)	33.2 ± 26.1 25.5 (10.8-188.0)	0.58
E1	3.0 ± 2.8 2.4 (1.2-17.7)	2.8 ± 1.1 2.7 (1.2-5.5)	2.9 ± 2.2 2.5 (1.2-17.7)	0.97
E2	0.5 ± 0.2 0.5 (0.3-1.5)	0.5 ± 0.1 0.4 (0.3-0.7)	0.5 ± 0.2 0.5 (0.3-1.5)	0.23

* means there is significant sex difference ($p < 0.001$).

5-Pregnene-3β,21-diol-20-one (21 − OH-P5olon) (43.9 ± 24.0 ng/mL) showed the highest levels within this group. No significant sex differences were found for the sum of pregnenolone metabolites, the sum of pregnenolone and 17 − OH-pregnenolone metabolites and for individual metabolites.

In the group of DHEA and its metabolites, 16α − OH-DHEA (42.4 ± 21.8 ng/mL) and 16β − OH-DHEA (32.9 ± 30.3 ng/mL) were the two dominant steroids. No significant sex differences were found for DHEA and its metabolites.

The predominant steroid in the group of progesterone and 17 − OH-progesterone metabolites was pregnanediol (91.6 ± 41.0 ng/mL). The concentrations of pregnanediol were also the highest of all measured individual steroid hormone metabolites in AF samples.

In the group of sex hormones, testosterone showed a highly significant sex difference ($p < 0.0001$). E3 (33.2 ± 26.1 ng/mL) was by far the primary estrogen metabolite.

Corticosterone metabolites (THA, α-THB and THB) and 11-deoxycortisol metabolites (THS) were present in rather low concentrations in AF samples. Cortisol metabolites such as THF, α-THF, 6β − OH-F, α-Cortol, β-Cortol and 20α-DHF were all present in rather low concentrations. No significant sex differences were found. The levels of THE were the highest of all measured cortisone and cortisol metabolites. The sum of cortisone metabolites was clearly higher than the sum of cortisol metabolites.

4. Discussion

The ionization of steroids lacking the 3-oxo-Δ^4 unconjugated ring system by soft ionization techniques, such as electrospray ionization or atmospheric pressure chemical ionization, applied in LC–MS/MS is problematic. Steroids bearing a 3β-hydroxy-5-ene or saturated ring structure are difficult to ionize and thus might even not be amenable to analysis by LC–MS/MS. However, electron impact ionization used with GC–MS overcomes this problem. Thus, GC–MS is more suitable for providing an integrated picture of the steroid metabolome. Furthermore, GC–MS has the advantage over LC–MS/MS of better chromatographic resolution and sensitivity. Consecutively, GC–MS has proved to be a powerful tool in the delineation of steroid metabolomes and the diagnosis of clinical disorders [[17,18]]. However, in contrast to LC–MS/MS, GC–MS does not allow for the determination of intact steroid conjugates [19]. Therefore, hydrolysis of conjugates is required to remove the conjugate moiety, i.e. sulfate, glucuronide. For this reason, the concentrations reported in the present study resemble total concentrations of free and conjugated steroids [20]. Hill et al. [21] also investigated steroids in AF. However, AF samples from late pregnancy had been analyzed, and a different sample workup procedure had been used.

4.1. Pregnenolone and 17 − OH-pregnenolone metabolites

In steroid hormone biosynthesis, pregnenolone, a C_{21} steroid unsaturated at $C_{5\text{-}6}$ presents the first steroid hormone. Along the so called Δ5 pathway, it is further converted to the Δ5 steroids, 17 − OH-pregnenolone (C_{21} steroid) and DHEA (C_{19} steroid). Steroids bearing the structure "3β-hydroxy-5-pregnene" are metabolites of pregnenolone such as P5D, 21 − OH-P5olon, 16α − OH-P5olon, P5-3β,20α,21-triol and P5-tetrol. Compounds revealing the structure "3β,17α-dihydroxy-5-pregnene" are metabolites of 17 − OH-pregnenolone such as P5T-17α, 15β,17α − OH-P5olon and P5-tetrol-15β. The sum of pregnenolone metabolites and 17 − OH-pregnenolone metabolites showed the highest concentration of all groups of steroids investigated (Tables 1a and 1b). No significant sex differences were found for pregnenolone metabolites and Δ5 unsaturated C_{21}steroids.

Pregnenolone and 17 − OH-pregnenolone undergo various hydroxylations in various positions. 16α-hydroxylase is intensely active in mid-term fetal liver [30]. Pregnenolone and P5-3β,20α,21-triol can both be hydroxylated at 16α-position yielding 16α − OH-P5olon and P5-tetrol, respectively. 21-hydroxylase is also active in mid-term fetal adrenal cortex with 21 − OH-P5olon as its product [30].

A high activity in 20α-hydroxysteroid dehydrogenase (20α-HSD) is reflected by the conversion of 21 − OH-P5olon to P5-3β,20α,21-triol, and of 15β,17α − OH-P5olon to P5-tetrol-15β. The concentration levels of P5-3β,20α,21-triol and 21 − OH-P5olon were the highest of all pregnenolone and 17 − OH-pregnenolone metabolites.

4.2. DHEA and metabolites

"3β-hydroxy-5-androstene" C_{19} steroids are DHEA metabolites and weak androgens. They form the group with the second highest steroid hormone concentrations. No significant sex difference was found for the sum of DHEA and its metabolites.

The highest concentrations of DHEA metabolites were found for 16α − OH-DHEA, 16β − OH-DHEA and DHEA. DHEAS is produced by the fetal adrenal, then is 16α-hydroxylated in the fetal liver to yield large amounts of 16α − OH-DHEAS, the precursor of E3.

16β − OH-DHEA is also typically present in the urine of newborns and infants. 16α-hydroxylase has been researched in the feto-placental unit substantially, while activity of 16β-hydroxylase in fetuses has so far been unknown [30]. The comparable concentrations of 16β − OH-DHEA with 16α − OH-DHEA in AF suggest that 16β-hydroxylase is also active in the feto-placental unit.

In our previous paper [12], we found a strong correlation between DHEAS and 16α − OH-DHEAS measured intact in AF by LC–MS/MS. However, no correlations were found for total DHEA and total16α − OH-DHEA as well as total DHEA and total 16β − OH-DHEA in the present study. This illustrates that DHEA might not be the only

Table 1b
Concentrations of corticosterone and cortisol metabolites.

Steroids	Concentrations (ng/mL)			p value (male & female)
	Male	Female	Total	
Corticosterone metabolites (C_{21} steroids)				
THA	1.2 ± 1.5	1.2 ± 1.0	1.2 ± 1.3	0.24
	1.4 (0.0-5.9)	1.7 (0.0-2.6)	1.6 (0.0-5.9)	
α-THB	0.8 ± 1.1	0.3 ± 0.7	0.6 ± 1.0	0.53
	0.0 (0.0-2.9)	0.0 (0.0-2.4)	0.0 (0.0-2.9)	
THB	0.0 ± 0.0	0.9 ± 4.7	0.4 ± 3.2	–
	0.0 (0.0-0.0)	0.0 (0.0-26.0)	0.0 (0.0-26.0)	
11-deoxycortisol metabolite (C_{21} steroids)				
THS	0.1 ± 0.4	0.1 ± 0.5	0.1 ± 0.4	–
	0.0 (0.0-2.4)	0.0 (0.0-2.6)	0.0 (0.0-2.6)	
F and metabolites (C_{21} steroids)				
F	8.7 ± 2.2	8.1 ± 2.4	8.4 ± 2.3	0.07
	9.0 (0.0-12.0)	7.8 (2.0-16.1)	8.5 (0.0-16.1)	
THF	4.8 ± 1.7	3.8 ± 1.3	4.4 ± 1.6	0.04
	4.6 (2.4-8.2)	3.7 (0.9-7.3)	4.0 (0.9-8.2)	
α-THF	4.4 ± 1.7	3.5 ± 1.9	4.0 ± 1.9	0.10
	4.2 (0.0-10.1)	3.5 (0.0-8.4)	4.0 (0.0-10.1)	
6β-OH-F	3.4 ± 0.7	3.1 ± 0.9	3.3 ± 0.8	0.25
	3.3 (2.1-5.3)	2.9 (0.0-5.0)	3.2 (0.0-5.3)	
α-Cortol	0.8 ± 0.7	0.8 ± 0.6	0.8 ± 0.6	0.04
	1.2 (0.0-1.9)	1.0 (0.0-1.9)	1.1 (0.0-1.9)	
β-Cortol	1.5 ± 0.5	1.1 ± 0.6	1.3 ± 0.6	0.08
	1.3 (0.9-2.9)	1.1 (0.0-2.7)	1.2 (0.0-2.9)	
20α-DHF	1.8 ± 0.5	1.8 ± 0.6	1.8 ± 0.5	0.71
	1.8 (0.9-3.1)	1.7 (0.0-3.6)	1.7 (0.0-3.6)	
Σ F and metabolites	25.5 ± 4.9	22.2 ± 5.1	24.1 ± 5.2	0.09
	24.8 (7.2-35.1)	22.3 (3.0-30.1)	24.0 (3.0-35.1)	
E (C_{21} steroids)				
THE	18.8 ± 4.5	16.7 ± 3.5	18.0 ± 4.2	0.06
	17.8 (12.6-34.1)	15.7 (10.1-25.0)	17.0 (10.1-34.1)	
α-Cortolone	2.3 ± 3.9	1.6 ± 2.6	2.0 ± 3.4	0.61
	0.0 (0.0-17.5)	0.0 (0.0-6.8)	0.0 (0.0-17.5)	
β-Cortolone	2.7 ± 0.9	2.3 ± 1.0	2.6 ± 0.9	0.07
	2.4 (1.6-4.9)	2.2 (0.5-4.0)	2.3 (0.5-4.9)	
6α-OH-THE	2.7 ± 1.0	2.6 ± 0.9	2.7 ± 0.9	0.83
	2.6 (1.3-6.0)	2.6 (0.7-4.7)	2.6 (0.7-6.0)	
6α-OH-β-Cl	6.9 ± 3.1	6.2 ± 2.9	6.7 ± 3.0	0.53
	6.3 (0.0-13.5)	6.4 (0.0-13.5)	6.3 (0.0-13.5)	
6α-OH-α-Cl	5.5 ± 2.1	5.6 ± 3.4	5.6 ± 2.8	0.52
	5.1 (1.4-11.7)	4.4 (0.5-14.8)	4.9 (0.5-14.8)	
1β-OH-THE	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	–
	0.0 (0.0-0.3)	0.0 (0.0-0.4)	0.0 (0.0-0.4)	
1β-OH-β-Cl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–
	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	
Σ E metabolites	36.7 ± 9.5	33.5 ± 9.8	35.5 ± 9.6	0.24
	37.4 (16.9-68.6)	35.8 (11.8-52.7)	34.9 (11.8-68.6)	
(Σ F + Σ E) metabolites	62.2 ± 13.4	55.7 ± 13.4	59.6 ± 13.6	0.13
	61.2 (24.1-103.8)	57.7 (14.8-81.6)	60.2 (14.8-103.8)	

precursor of 16α − OH-DHEA and 16β − OH-DHEA in the feto-placental unit. We speculate that 16α − OH-P5olon is the main precursor of 16α − OH-DHEA and 16β − OH-DHEA. This was also corroborated by the strong correlation between 16α − OH-P5olon and 16α − OH-DHEA ($r = 0.83$, $p < 1.00 \times 10^{-14}$), 16α − OH-P5olon and 16β − OH-DHEA ($r = 0.76$, $p = 1.42 \times 10^{-13}$), respectively. Following the Δ5 pathway, 16α − OH-P5olon is metabolized to 16α,17α − OH-pregnenolone by 17α-hydroxylase, then it is converted to 16α − OH-DHEA by 1720 lyase.

A strong correlation was found between 16α − OH-DHEA and 16β − OH-DHEA ($r = 0.88$, $p < 1.00 \times 10^{-14}$). There are two possible explanations: they might either stem from the same precursor or a hydroxysteroid epimerase (HSE) that converts the 3-hydroxy group between α and β positions [22]. Likewise, a similar enzyme can act at position 16 to interconvert 16α − OH-DHEA and 16β − OH-DHEA. 16-O-A5D is an oxidized product of 16α − OH-DHEA and 16β − OH-DHEA. A strong correlation with 16α − OH-DHEA ($r = 0.86$, $p < 1.00 \times 10^{-14}$) points to the former compound as its main metabolic source.

DHEA is metabolized to A5-3β,17α and A5-3β,17β by 17α-HSD and 17β-HSD, respectively. A good correlation was only found between DHEA and A5-3β,17β ($r = 0.69$, $p = 1.99 \times 10^{-8}$), however not between DHEA and A5-3β,17α ($r = 0.34$, $p = 0.007$). Research in rabbits shows that 17α-HSD acts more slowly on the 17α-hydroxy group of androgenic substrates [23].

4.3. Progesterone and 17 − OH-progesterone metabolites

"3α-hydroxy-5-pregnane" steroids without a 21-hydroxy group are metabolites of progesterone or 17 − OH-progesterone. In this group, pregnanediol is by far the most dominating metabolite. No sex difference was found for progesterone and 17 − OH-progesterone metabolites.

4.4. Other androgens

Testosterone and "3α-hydroxy-5-androstane" steroids belong to the class of androgens (C_{19} steroids). Only testosterone showed a highly significant sex difference in AF samples ($P < 0.0001$), which is consistent with previous findings in the literature [[24,25]]. Our results indicate that female and male fetuses both produce testosterone in feto-placental unit. We speculate that the higher levels of testosterone are due to the intrauterine activity of the fetal testis. Androsterone (An) and etiocholanolone (Et) are both present in low concentrations in AF. 5α-androstane-3α,11β-diol-17-one (11 − OH-An) and 5α-androstane-3α-ol-11,17-dione (11-O-An) are metabolites of androstenedione.

4.5. Backdoor pathway

In the classic Δ5 steroid pathway, 17-hydroxy-pregnenolone is metabolized to DHEA and then converted to androstenedione and testosterone. In the so called alternative "backdoor" pathway, 17-hydroxy-progesterone is metabolized to 17α-hydroxy-dihydroprogesterone (5α-pregnane-17α-ol-3,20-dione) via 5α-reductase and then converted to 17α-hydroxy-allopregnanolone (5α-Pregnane-3α,17α-diol-20-one, Po-5α,3α) by 3α-hydroxysteroid dehydrogenase (3α-HSD). 17α-hydroxy-allopregnanolone is the precursor of An. An is then metabolized to dihydrotestosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3α-HSD [22]. It is assumed that both the classic and alternative "backdoor" pathways are involved in normal human male sexual differentiation [26].

The An to Et ratio can be used as an indicator for the activity of the alternative "backdoor" pathway. Furthermore, the ratio between 17α-hydroxy-allopregnanolone and 11 − OH-An can be used to calculate the activity of "backdoor" pathway vs. that of the Δ4 pathway [27]. In our study, no significant sex differences were found for both ratios ($p = 0.71$; $p = 0.38$). 17α-hydroxy-allopregnanolone/17α-hydroxy-pregnanolone (Po-5β,3α) and α-THF/THF are used to calculate the activity of 5α-reductase activity [27]. Likewise, no significant sex differences were found for these ratios ($p = 0.24$; $p = 0.93$). We thus conclude that the hormonal constellation in AF of mid-gestation was not indicative of an active "backdoor" pathway.

4.6. Estrogens

E3 presented by far the dominant estrogen. It is produced mainly in placenta by conversion of 16α − OH-DHEA with which it shows positive correlation ($r = 0.54$, $p = 3.09 \times 10^{-6}$). E3, the main estrogen in the feto-placental unit, is a good marker for fetal adrenal and adrenocorticotropic functions. Low maternal E3 is a hallmark of diseases such as aromatase deficiency, Smith-Lemli-Opitz Syndrome, steroid sulfatase deficiency, P450 oxidoreductase deficiency or severe neonatal adrenal insufficiency [2].

4.7. Cortisol and metabolites

The transitional zone of the fetal adrenal expresses 17α-hydroxylase and 3β-HSD2 to produce cortisol *de novo* from the second half of gestation onwards [8]. We found cortisone and cortisol metabolites to be clearly present in our AF samples of mid-gestation (as shown in Table 1b). A possible explanation for a source of cortisol in AF of mid-gestation could be the maternal transfer of cortisol across the placenta. Another reason could be placental progesterone acting as a precursor of cortisol production in the fetus [30]. A third possibility could be an earlier start of fetal *de novo* cortisol biosynthesis than hitherto believed.

The interconversion between cortisone and cortisol is catalyzed by a shuttle system consisting of two distinct enzymes: 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [28]. 11β-HSD2 converts active cortisol to inactive cortisone and is mainly localized in kidneys and placenta, whereas 11β-HSD1 converts cortisone to cortisol and is present in liver and fat tissue [29]. THE and THF are important tetra-hydrogenated metabolites of cortisone and cortisol, respectively. They show a strong correlation ($r = 0.90$, $p < 1.00 \times 10^{-14}$). β-Cortol and β-Cortolone are hexahydrated metabolites of THE and THF, respectively. They also showed positive correlation ($r = 0.67$, $p = 3.69 \times 10^{-9}$). We found the concentrations of cortisone metabolites to be clearly higher than those of cortisol metabolites in our study. This points to a preference of cortisol inactivation by the feto-placental unit. By LC–MS/MS, Fahlbusch et al [25] also measured comparable cortisol concentrations in AF of mid-gestation (male: 7.34 ± 3.01 ng/mL, female: 6.38 ± 2.73 ng/mL, $p = 0.057$).

5. Conclusion

Steroid hormone metabolites in AF of mid-gestation were delineated by a targeted GC–MS approach. Pregnenolone and 17 − OH-pregnenolone metabolites, DHEA and metabolites, progesterone and 17 − OH-progesterone metabolites, androgens, estrogens as well as cortisone and cortisol metabolites were characterized in AF of mid-gestation. Reference data of levels of 52 steroids in AF of mid-gestation were provided in the present study. These reflected that all important classes of steroids are present in AF of mid-gestation. This set of basic data might lay the foundation for further studies characterizing various diseases affecting steroid metabolism.

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Appendix A. Supplementary data

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2.3 Publication 3

Targeted LC-MS/MS analysis of steroid glucuronides in human urine.

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Abstract

We have developed and validated a LC-MS/MS method for the simultaneous quantification of 15 urinary steroid glucuronides in human urine: androsterone glucuronide, etiocholanolone glucuronide, epiandrosterone glucuronide, dihydrotestosterone glucuronide, dehydroepiandrosterone glucuronide, testosterone glucuronide, epitestosterone glucuronide, estrone glucuronide, 17β-estradiol 17-glucuronide, 17β-estradiol 3-glucuronide, estriol 16-glucuronide, pregnenolone glucuronide, Tetrahydro-11-deoxycorticosterone 3glucuronide, cortisol 21-glucuronide and pregnanediol glucuronide. The method was then successfully applied to 67 urine samples from 5-day-old infants to 17.8-year-old children and adolescents. Free and sulfated steroids were also measured by LC-MS/MS. In addition, GC-MS measurements provided a total concentration of free and conjugated steroids after enzymatic hydrolysis. The total levels of androsterone and etiocholanolone went up to 5820.0 nmol/L and 4017.8 nmol/L through childhood, respectively. They are largely excreted as glucuronides (4374.3 nmol/L and 3588.5 nmol/L, respectively). DHEA was excreted mostly as sulfate in all age groups. Cortisol was present predominantly as sulfate (173.8 nmol/L) in newborns. The levels of sulfated cortisol deceased with age while its glucuronidated form increased. The levels of free cortisol were relatively constant in the urine of children after 1 year of age. Sex hormones were mainly excreted as glucuronide. This study for the first time showed the distribution profile of steroids in free, sulfated and glucuronidated forms.

Contribution

The first author carried out the study according to the advice of her supervisor. The first author performed and developed procedures for sample workup, instrumental analysis and data processing. The first author created all the figures and tables for the manuscript and drafted the manuscript. The drafted manuscript was reviewed, supplemented and corrected together with all other co-authors.

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Targeted LC–MS/MS analysis of steroid glucuronides in human urine

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ABSTRACT

Conjugation with glucuronic acid is one of the major metabolic reactions in human steroid hormone catabolism. Recently, increasing interest has been raised concerning the biological roles of steroid glucuronides. We have therefore developed and validated a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of 15 urinary steroid hormone glucuronides in human urine: androsterone glucuronide (An-G), etiocholanolone glucuronide (Etio-G), epiandrosterone glucuronide (epiAn-G), dihydrotestosterone glucuronide (DHT-G), dehydroepiandrosterone glucuronide (DHEA-G), testosterone glucuronide (T-G), epitestosterone glucuronide (epiT-G), estrone glucuronide (E1−3 G), 17β-estradiol 17-glucuronide (E2−17 G), 17β-estradiol 3-glucuronide (E2−3 G), estriol 16-glucuronide (E3−16 G), pregnenolone glucuronide (Preg-G), tetrahydro-11-deoxycorticosterone 3-glucuronide (THDOC-3 G), cortisol 21-glucuronide (F–G) and pregnanediol glucuronide (PD-G). Sample workup included protein precipitation and solid phase extraction. Internal standards were used to correct for the loss of analytes during sample preparation and analysis. The method showed good linearity ($R^2 \geq 0.99$) and recovery ranged from 89.6 % to 113.8 %. Limit of quantification ranged from 1.9 nmol/L for F–G to 21.4 nmol/L for An-G. Intra-day and inter-day accuracy and precision were below 15 % for all quality controls. The method was successfully applied to 67 urine samples from children and adolescents in whom total concentrations of free and conjugated steroids had been previously determined by GC–MS after enzymatic hydrolysis. Free and sulfated steroids were also measured by LC–MS/MS. In general, the sums of the respective glucuronidated, sulfated and free forms of an analyte corresponded well with its total amount determined after enzymatic hydrolysis by GC–MS. Regarding the most prominent steroid metabolites, the total mean levels of androsterone and etiocholanolone showed an increase up to 5820.0 nmol/L and 4017.8 nmol/L in the group of 15−20 year-old children, respectively. Glucuronide conjugates (4374.3 nmol/L and 3588.5 nmol/L, respectively) dominated. DHEA was excreted mostly as sulfate (0−1 month of age: 184.5 nmol/L; 15−20 years of age: 1618.4 nmol/L) in all age groups. Cortisol was present predominantly as sulfate (mean: 173.8 nmol/L) in newborns. Levels of sulfated cortisol decreased with age, its glucuronidated form increased. The levels of free cortisol were relatively constant throughout childhood. Sex hormones were preferably excreted

Abbreviations: LC–MS/MS, liquid chromatography-tandem mass spectrometry; UGTs, Uridine diphos-phoglucuronosyl transferases; MRP, multidrug resistance protein; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; UHPSFC-MS/MS, ultra-high-performance supercritical-fluid chromatography-tandem mass spectrometry; epiAn-G, 5α-androstane-3β-ol-17-one glucuronide (epiandrosterone glucuronide); DHT-G, 5α-androstane-17β-ol-3-one glucuronide (dihydrotestosterone glucuronide); Etio-G, 5β-androstane-3α-ol-17-one glucuronide (etiocholanolone glucuronide); DHEA-G, 5-androstene-3β-ol-17-one glucuronide (dehydroepiandrosterone glucuronide); F-G, 4-pregnene-11β,17,21-triol-3,20-dione 21-glucuronide (cortisol 21-glucuronide); E1-G, 1,3,5(10)-estratriene-3-ol-17-one 3-glucuronide (estrone 3-glucuronide); E2-17G, 1,3,5(10)-estratriene-3,17β-diol 17-glucuronide (17β-estradiol 17-glucuronide); E2-3G, 1,3,5(10)-estratriene-3, 17β-diol 3-glucuronide (17β-estradiol 3-glucuronide); Preg-G, 5-pregnene-3β-ol-20-one glucuronide (pregnenolone glucuronide); An-G, 5α-androstane-3α-ol-17-one glucuronide (androsterone glucuronide); T-G, 4-androstene-17β-ol-3-one glucuronide (testosterone glucuronide), epiT-G, 4-androstene-17α-ol-3-one glucuronide (epitestosterone glucuronide); d_3DHT-G, [16,16,17-d_3]5α-androstane-17β-ol-3-one glucuronide (d_3-dihydrotestosterone glucuronide); d_4An-G, [2,2,4,4-d_4]5α-androstane-3α-ol-17-one glucuronide (d_4-androsterone glucuronide); E3-16G, 1,3,5(10)-estratriene-3,16α,17β-triol 16-glucuronide (estriol 16-glucuronide); d_3E2-3G, [16,16,17-d_3]13,5(10)-estratriene-3, 17β-diol 3-glucuronide ([16,16,17-d_3]17β-estradiol 3-glucuronide); d_3T-G, [16,16,17-d_3]4-androstene-17β-ol-3-one glucuronide [16,16,17-d_3]testosterone glucuronide); $d_4$epiT-G, [1,16,16,17-d_4]4-androstene-17α-ol-3-one glucuronide [1,16,16,17-d_4]epitestosterone glucuronide); THDOC 3-G, 5β-pregnane-3α,21-diol-20-one 3-glucuronide (tetrahydro-11-deoxycorticosterone glucuronide); PD-G, 5β-pregnane-3α,20α-diol 3-glucuronide (pregnanediol 3-glucuronide); MeOH, methanol; ACN, acetonitrile; LOQ, Limit of quantification; LOD, Limit of detection; QC, quality control.

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as glucuronides. In general, steroid hormone metabolites were conjugated to various extents with glucuronic acid or sulfuric acid and their ratio changed over lifetime.

1. Introduction

Human steroids are mainly synthesized in endocrine glands such as gonads and adrenal glands as well as in liver or placenta [1]. They undergo phase I metabolism including oxidation, reduction or hydroxylation and phase II metabolism to form more polar compounds such as sulfated or glucuronidated conjugates [2]. Glucuronidation is an important pathway in mammals. Uridine diphosphoglucuronosyl transferases (UGTs) are the enzymes required for glucuronidation, while β-glucuronidases catalyze the hydrolysis of steroid glucuronides by cleaving the β-glucuronic acid (Fig. 1) [3]. Human liver is the main site for glucuronidation [4]. However, conjugation of steroids can also take place in other organs such as kidney, brain and prostate [5].

For a long time it has been believed that steroids present only in their free unconjugated form can interact with receptors and exert biological effects [6]. However, e.g. for androgen glucuronides it has meanwhile been found that they can also be deconjugated by multidrug resistance protein (MRP) in human gut lumen thus influencing the physiological levels of androgens [7]. Furthermore, estrogen glucuronides can also be transported in vitro by human MRP using vesicular transport assay. MRP3 transported estrone glucuronide (E1−3 G), estradiol 3-glucuronide (E2−3 G), estriol 3-glucuronides (E3−3 G) and estriol 16-glucuronides (E3−16 G) at rather high affinity, whereas MRP4 only transported E3−16G [8]. It has also been shown that the concentrations of androsterone glucuronide in serum might reflect effective androgenic activity in women [9]. Androsterone glucuronide levels in plasma were sensitive in detecting differences between control and acne groups presenting a promising biomarker for peripheral hyperandrogenism in adult female acne patients [10].

The glucuronidation and deglucuronidation reactions are reversible in the human body (Fig. 1). By cleavage of the conjugate, steroid glucuronides can be transformed to their active free forms and then execute biological functions. In many studies dealing with drug metabolism or toxicology, it is necessary to determine glucuronide metabolites [11]. Due to the water solubility of the glucuronic acid functional group, higher concentrations of steroid glucuronides are present in urine, compared to plasma or serum [12].

Excretion of urinary steroids is directly related to age and sex. Insufficient or excessive excretion suggests abnormal enzymatic activities of UGTs and β-glucuronidase. However, hardly any information is available concerning the physiological levels of intact steroid glucuronides in human body fluids, e.g. urine. Therefore, an analytical method for reliable measurement was required for determining urinary concentrations of steroid glucuronides.

Due to soft ionization, LC–MS allows quantifying the intact steroid conjugate and is therefore the technique of choice for these compounds in steroid analysis [13]. It avoids a hydrolysis step in sample preparation. Our group has successfully demonstrated the applicability of LC–MS to the analysis of steroid sulfates [14]. To date, only a few groups have investigated steroid glucuronides in human body fluids by MS based analytical techniques. Table 1 summarizes the findings for the most commonly determined steroid glucuronides, i.e. androgen glucuronides. However, concentrations have not been reported [15,16] and validation data are sometimes missing [2,12,17]. Therefore, we have developed and fully validated a new method on the basis of LC–MS/MS to simultaneously quantify steroid glucuronides in human urine.

2. Materials and methods

2.1. Chemicals

Commercially available steroid glucuronides were obtained from various suppliers. 5α-androstane-3β-ol-17-one glucuronide (epiandrosterone glucuronide, epiAn-G), 5α-androstane-17β-ol-3-one glucuronide (dihydrotestosterone glucuronide, DHT-G), 5β-androstane-3α-ol-17-one glucuronide (etiocholanolone glucuronide, Etio-G), 5-androstene-3β-ol-17-one glucuronide (dehydroepiandrosterone glucuronide, DHEA-G), 4-pregnene-11β,17,21-triol-3,20-dione 21-glucuronide (cortisol 21-glucuronide, F–G), 1,3,5(10)-estratriene-3-ol-17-one 3-glucuronide (estrone 3-glucuronide, E1−3 G), 1,3,5(10)-estratriene-3, 17β-diol 17-glucuronide (17β-estradiol 17-glucuronide, E2−17 G), 1,3,5(10)-estratriene-3,17β-diol 3-glucuronide (17β-estradiol 3-glucuronide, E2−3 G) and 5-pregnene-3β-ol-20-one glucuronide (pregnenolone glucuronide, Preg-G) were purchased from Steraloids Inc. (Newport, RI). 5α-androstane-3α-ol-17-one glucuronide (androsterone glucuronide, An-G), 4-androstene-17β-ol-3-one glucuronide (testosterone glucuronide, T-G), 4-androstene-17α-ol-3-one glucuronide (epitestosterone glucuronide, epiT-G), [16,16,17-d_3]5α-androstane-17β-ol-3-one glucuronide (d_3-dihydrotestosterone glucuronide, d_3DHT-G) and [2,2,4,4-d_4] 5α-androstane-3α-ol-17-one glucuronide (d_4-androsterone glucuronide, d_4An-G) were obtained from LGC Standards GmbH (Wesel, Germany). 1,3,5(10)-estratriene-3,16α,17β-triol 16-glucuronide (estriol 16-glucuronide, E3−16 G) was bought from Sigma-Aldrich (Taufkirchen, Germany). [16,16,17-d_3]1,3,5(10)-estratriene-3, 17β-diol 3-glucuronide ([16,16,17-d_3]17β-estradiol 3-glucuronide, d_3E2−3 G) and [16,16,17-d_3]4-androstene-17β-ol-3-one glucuronide ([16,16,17-d_3]testosterone glucuronide, d_3T-G) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). [1,16,16,17-d_4]4-androstene-17α-ol-3-one glucuronide ([1,16,16,17-d_4]epitestosterone glucuronide, $d_4$epiT-G) was bought from BDG synthesis Limited (Wellington, New Zealand). 5β-pregnane-3α,21-diol-20-one 3-glucuronide (tetrahydro-11-deoxycorticosterone 3-glucuronide, THDOC-3 G) and 5β-pregnane-3α,20α-diol 3-glucuronide (pregnanediol 3-glucuronide, PD-G) were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

Water (LC–MS grade), ammonium hydroxide and formic acid were purchased from Fluka (Taufkirchen, Germany). Methanol (MeOH), acetonitrile (ACN), n-hexane, and chloroform were obtained from Merck (Darmstadt, Germany). Ammonium acetate was from Sigma-Aldrich.

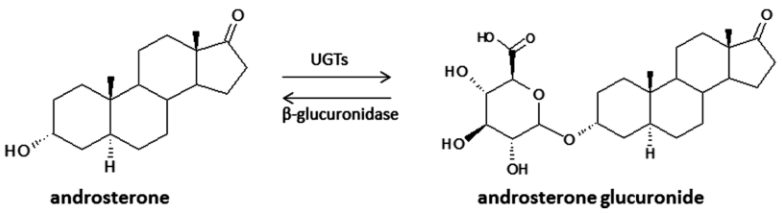

Fig. 1. Formation of androsterone glucuronide by UGTs (uridine diphosphoglucuronosyl transferases) and deconjugation by β-glucuronidase in the human body.

SepPak C18 cartridges (200 mg) were from Waters Corporation (Milford, MA), zinc sulfate was from Roth (Karlsruhe, Germany).

2.2. Creatinine measurement

Creatinine was determined by ADVIA Chemistry XPT-System (Simens, Germany). Urine samples were centrifuged (3800 *g*, 3 min) whenever precipitation was observed. Then 200 μL of urine was used for measurement. The results were expressed as milligrams per dl urine.

2.3. Sample preparation

2 mL of urine were incubated with 100 μL of a cocktail containing all internal standards (IS). Regarding newborns, 200 μL of urine were mixed with 10 μL of the IS cocktail. The concentration of each internal standard added was 2.5 μg/mL. The samples were mixed well with IS by shaking for 20 min on a shaker. 1 mL of a solution consisting of acetonitrile-ZnSO4 [89 g/L, 4:1 (v/v)] was added to each sample for protein precipitation. The samples were shaken again for 15 min and centrifuged for 10 min at 3800 *g*. The supernatant was transferred to a glass tube containing 3 mL of water and then mixed. SepPak cartridges were activated with 3 mL of methanol and 3 mL of water. The solution in the glass tube was loaded on the cartridge and washed with 3 mL of water to remove water soluble interferences. Then 3 mL of hexane were used to wash highly lipophilic substances from the column. With 4 mL of chloroform, unconjugated steroids were eluted. Then, steroid glucuronides as well as sulfates were extracted by eluting with 4 mL of methanol. Thereafter the methanolic fraction was evaporated at 40 °C under a stream of nitrogen and reconstituted with 200 μL of a solution consisting of 79.75 % water, 10 % MeOH, 10 % ACN and 0.25 % ammonium hydroxide. Centrifugation was used to remove undissolved substances whenever precipitation was observed. 20 μL of the final extract were injected for the analysis by LC–MS/MS.

2.4. Liquid chromatography

LC separation was achieved on an Acclaim™ C30 column (100 × 2.1 mm, 3 μm, Thermo Fisher Scientific, Dreieich, Germany). The LC system was an Agilent 1200SL (Waldbronn, Germany). Mobile phase A consisted of 10 mM ammonium acetate buffer (pH = 7 adjusted by ammonium hydroxide). Mobile phase B consisted of a mixture of MeOH and ACN [3:7 (v/v)]. The LC gradient condition is presented in Table 2.

2.5. Mass spectrometry

A triple quadrupole mass spectrometer (TSQ, Quantum Ultra, Thermo Scientific, Dreieich, Germany) with an electrospray probe in negative mode was utilized for detection and quantification of steroid glucuronides. The capillary temperature and the vaporizer temperature were 250 °C and 300 °C, respectively. The sheath gas and arbitrary gas were set at 50 arbitrary units and 20 arbitrary units, respectively. The voltage applied was 3500 V.

Table 2
Flows and solvent percentages for the LC method.

Step	Second	Flow(mL/min)	Solvent A (%)	Solvent B (%)
1	1	0.35	75	25
2	720	0.35	60	40
3	240	0.35	30	70
4	120	0.35	1	99
5	10	0.35	75	25
6	180	0.35	75	25

Table 1
Steroid glucuronides commonly determined by mass spectrometric methods.

Steroids	Medium		Method	Method validation data	Physiological concentrations (ng/mL)from healthy adults	Year	Reference
An-G	urine	human	LC-MS/MS	No		1996	[12]
			UHPLC-QTOF-MS	Yes		2011	[16]
			GC-MS	Yes	512–15588 (n = 15)	2008	[18]
			GC-MS	Yes	2249.6 1874.7–3124.6 (n = 5 males)	2000	[19]
	plasma	human	LC-MS	Yes		2016	[20]
	serum	human	UPLC–MS/MS	Yes		2015	[15]
Etio-G	urine	human	LC-MS/MS	No		1996	[12]
			UHPLC-QTOF-MS	Yes		2011	[16]
			GC-MS	Yes	411–9499 (n = 15)	2008	[18]
			GC-MS	Yes	1892.8 1569.2–2855.2 (n = 5 males)	2000	[19]
		bovine	UHPSFC-MS/MS	Yes		2015	[21]
			UPLC–MS/MS	No		2011	[17]
	serum	human	UPLC–MS/MS	Yes		2015	[15]
DHEA-G	urine	human	LC-MS/MS	No		1996	[12]
			UHPLC-QTOF-MS	Yes		2011	[16]
			GC-MS	Yes	13–229 (n = 15)	2008	[18]
			GC-MS	Yes	49.2 32.2–50.9 (n = 5 males)	2000	[19]
		human during pregnancy	LC-MS/MS	No		2013	[2]
		bovine	UHPSFC-MS/MS	Yes		2015	[21]
			UPLC–MS/MS	No		2011	[17]
	tissue	mouse brain	LC-MS/MS	Yes	<3.7 (n = 1)	2010	[22]
T-G	urine	human	LC-MS/MS	No		1996	[12]
			UHPLC-QTOF-MS	Yes		2011	[16]
			GC-MS	Yes	1.1–137 (n = 15)	2008	[18]
			GC-MS	Yes	31.9 27.5–58.4 (n = 5 males)	2000	[19]
		human during pregnancy	LC-MS/MS	No		2013	[2]
		bovine	UHPSFC-MS/MS	Yes		2015	[21]
		bovine	UPLC–MS/MS	No		2011	[17]
	tissue	mouse brain	LC-MS/MS	Yes	<3.7 (n = 1)	2010	[22]

The concentrations were expressed as median and range if available.

2.6. Linearity, limit of quantification and limit of detection

The linearity of the method was evaluated by spiking different concentrations of standards and equal concentrations of internal standards into 2 mL of charcoal-stripped steroid-free urine. The stock solutions of standards and internal standards were prepared in methanol: water [1:1, (v/v)]. Linearity was calculated by plotting peak area ratios of calibration points against their corresponding concentrations. Calibration curves were established over the range of 21.4–10715.8 nmol/L for An-G, of 10.7–10715.8 nmol/L for Etio-G, of 4.0−1006.8 nmol/L for PDG, of 5.4−107.6 nmol/L for T-G, DHEA-G and epiT-G, of 5.4-1071.6 nmol/L for epiAn-G, of 5.4−107.6 nmol/L for DHT-G, of 5.6−111.5 nmol/L for E2−17 G, of 4.9–97.9 nmol/L for THDOC-3 G, of 20.3−1015.2 nmol/L for Preg-G, of 4.3–107.6 nmol/L for E3−16 G, of 4.5−111.5 nmol/L for E2−3 G, of 2.2−112.0 nmol/L for E1-G and of 1.9–185.7 nmol/L for F–G. Limit of quantification (LOQ) was defined as the lowest concentration at which the analytical system performs within a pre-specified imprecision (≤20 %, CV) and accuracy (100 % ± 20 %). Limit of detection (LOD) was determined at a signal-to-noise ratio higher than 3.

2.7. Quality controls

Quality controls (QCs) were prepared by spiking three different concentrations of each analyte into charcoal-stripped urine. The concentrations of QCs were selected according to the data found in the literature and the results from our preliminary experiments (Table 4).

2.8. Matrix effect and process efficiency

Matrix effect was evaluated by plotting response ratios of spiked charcoal-stripped urine after extraction with standards and internal standards (in the presence of matrix) against those of the directly prepared in reconstitution solution without sample workup (in absence of matrix). A slope equal to one implies no matrix effect. A slope above one indicates matrix enhancement, whereas a slope below one means matrix suppression.

Process efficiency (PE) is a quantitative overall assessment of matrix effect and impact of protein precipitation and extraction [23,24]. It was assessed by dividing response ratios of spiked standards and internal standards at each QC level into matrix with full sample preparation by those of standards and internal standards directly prepared in the reconstitution solution without sample preparation.

2.9. Recovery

Recovery was considered as extraction efficiency of the sample workup. The experiments were performed by comparing the response ratio of each QC with that of the same amounts of QCs prepared post-extraction (Recovery, % = response ratios obtained for spiked QCs / response ratios acquired from spiking the same amounts of the standards and IS post-extraction ×100).

2.10. Accuracy and precision

Intra-day accuracy and precision were assessed by analyzing five replicates of spiked QCs at three levels in a single sequence. Inter-day accuracy and precision were calculated by analyzing five replicates of QCs on each level on five different days. Individual accuracy was calculated by dividing measured concentrations of spiked QCs by known concentrations of spiked QCs. Accuracy was expressed as mean and standard deviation.

2.11. Freeze and thaw stability of samples

Two samples were analyzed 3 times to evaluate the freeze-thaw stability of samples. The samples were re-analyzed again one month and six months later after first time analysis. The samples were stored at −20 °C in a freezer until analysis.

2.12. Measurement of samples

As part of the evaluation process, our method was applied to 67 samples from children and adolescents in whom steroid related disorders had been excluded by previous routine GC–MS urinary steroid analysis (see below). In addition, unconjugated and sulfated steroids were measured by LC–MS/MS, as previously described [25,26]. Therefore, unconjugated steroids were eluted from solid phase extraction (SPE) cartridges with chloroform from cartridges and LC–MS/MS with APCI was used for instrumental analysis. Sulfated and glucuronidated steroids were eluted with methanol and both analyzed by LC–MS/MS with ESI. Regarding the GC–MS procedure, free and conjugated steroids were extracted by SPE from urine. After enzymatic hydrolysis, steroids were recovered by a second SPE step. Derivatization was conducted to form methyloxime-trimethylsilyl ethers. The derivatives were analyzed by a targeted GC–MS approach using selected ion monitoring mode [27].

2.13. Data analysis

Data analysis was made by Xcalibur 2.1 and Microsoft Excel 2010. Concentrations of steroids were shown as mean, standard deviation (SD), median as well as minimum and maximum. The concentrations measured below limit of detection (LOD) were treated as 0.0.

3. Results

3.1. Liquid chromatography/mass spectrometry

An Acclaim C30 LC column turned out to allow for sufficient resolution between all analytes and interferences. The chromatograms of analytes in a real sample and spiked standards in steroid-free matrix are shown in Fig. 2. The chromatograms clearly show that the LC method is capable of separating all isobaric analytes such as DHT-G/An-G/epiAn-G/Etio-G (m/z 465→113), T-G/epiT-G/DHEA-G (m/z 463→113), E2−3 G/E2−17 G (m/z 447→271).

Each standard was infused individually for acquiring MS transitions and parameters such as collision energy and tube lens (shown in Table 3). Then the mixture of all standards was injected to evaluate the optimal mass spectrometry conditions. Mass spectrometry parameters were studied under different conditions including spray voltage (−3000 V, −3500 V, −4000 V), vaporizer temperature (300 °C, 350 °C, 400 °C), capillary temperature (250 °C, 270 °C, 300 °C), sheath gas (40 psi, 50 psi, 60 psi) and auxiliary gas (10 psi, 20 psi). Best signals were found at -3500 V for spray voltage for all compounds, 300 °C for vaporizer temperature, 250 °C for capillary temperature, 50 arbitrary units for sheath gas and 20 arbitrary units for arbitrary gas.

3.2. Method validation

The method was validated in accordance with the US Food and Drug Administration and the EU European Medicines Agency guidelines for bioanalytical evaluation [28,29]. The validation results of the method have been summarized in Table 4. The method showed good linearity ($R > 0.99$) within the defined calibration range for all analytes. Calibration curves were shown in the supplementary data section. It had good accuracy (86.1 %–113.2 %) and precision (1.0 %–14.7 %). Recovery of all QCs ranged from 89.6%–113.8%. No significant matrix effects were observed for all steroid glucuronides, except THDOC-3 G, which showed mild ion suppression (0.83). Stability of the samples is shown in the supplementary data section. The variation of the concentrations of steroids for analyzed samples after six months was less than 15 %. It indicates that steroid glucuronides in urine samples are stable during

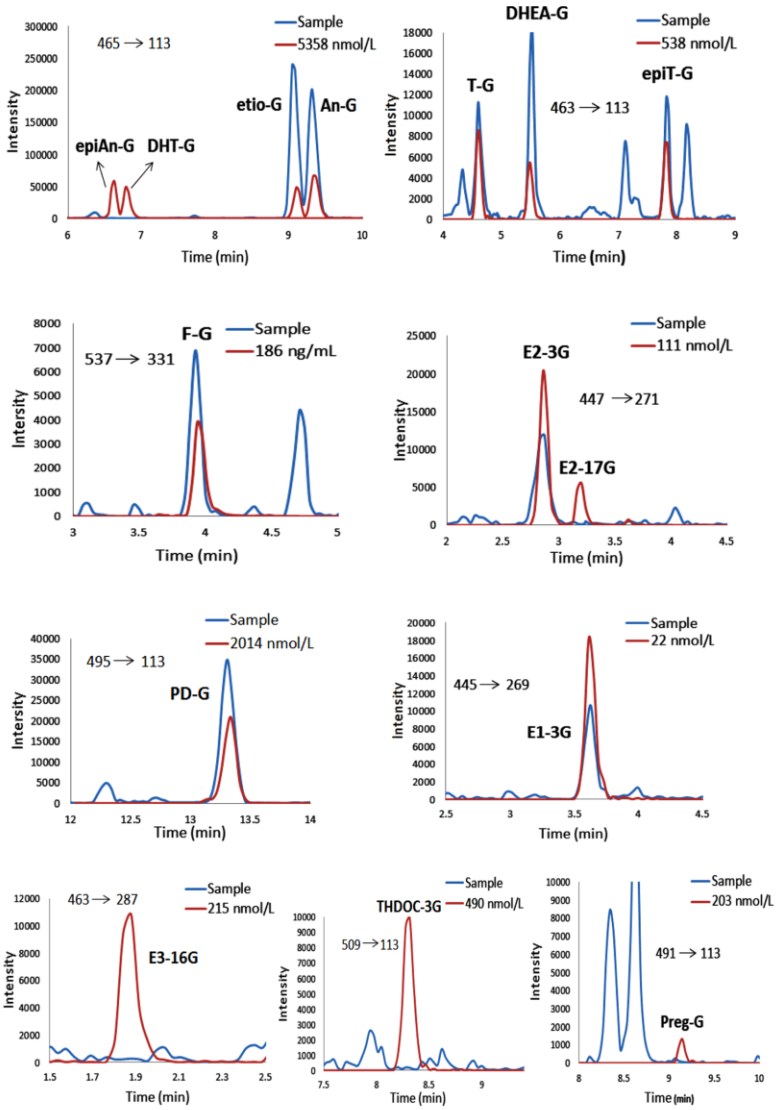

Fig. 2. The red graphs represent chromatograms of spiked standards of steroid glucuronides into steroid-free matrix. The blue graphs show chromatograms of steroid glucuronides in a urinary sample from an 18-year-old male.

long-term storage at −20 °C and no freeze-thaw effect was observed.

3.3. Free, sulfated and glucuronidated steroids in urine

The identification of an urinary analyte by LC–MS/MS was based on its retention time, mass transition as well as comparison with its corresponding stable isotope labeled internal standard if available. The concentrations of free, sulfated and glucuronidated steroids by LC–MS/MS and GC–MS were given in Table 5. The results of concentrations normalized by creatinine can be found in the supplementary data section. GC–MS analysis yielded total concentrations of free and conjugated steroids after enzymatic hydrolysis. During childhood and adolescence, the total mean levels of androsterone (An) and etiocholanolone (Etio) raised up to 5820.0 nmol/L and 4017.8 nmol/L in the group of 15−20 year-old children, respectively. LC–MS/MS analysis showed that by far the glucuronide fractions (4374.3 nmol/L and 3588.5 nmol/L, respectively) dominated. The total mean levels of DHEA (209.6 nmol/L) were high in newborns but decreased in infancy. Thereafter, mean DHEA levels increased again with age up to 2090.3 nmol/L in the group of 15−20 year-old children. DHEA was excreted mostly as sulfate in all age groups. The total concentrations of cortisol were relatively constant during the first 20 years of life. Cortisol was present predominantly as sulfate (mean: 173.8 nmol/L) in the first month of age. Thereafter, cortisol sulfate decreased to 36.6 nmol/L, whereas cortisol glucuronide increased up to 49.9 nmol/L in the age group of 15−20 year-old children. Sex hormones e.g. testosterone and estradiol were mainly excreted

Table 3
MS transition, fragments and parameters for steroids.

Compound	Parent ion fragment	Parent ion (m/z)	Product ion fragment	Product ion (m/z)	Collision Energy (eV)	Tube Lens (V)
An-G	$[M-H]^-$	465.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	31	109
epiAn-G	$[M-H]^-$	465.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	27	160
Etio-G	$[M-H]^-$	465.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	29	123
DHEA-G	$[M-H]^-$	463.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	26	113
DHT-G	$[M-H]^-$	465.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	26	157
T-G	$[M-H]^-$	463.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	27	114
epiT-G	$[M-H]^-$	463.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	30	118
E3−16G	$[M-H]^-$	463.2	$[M\text{-}H\text{-}Glu]^-$	287.1	33	96
E1-G	$[M-H]^-$	445.2	$[M\text{-}H\text{-}Glu]^-$	269.0	39	97
E2−3G	$[M-H]^-$	447.2	$[M\text{-}H\text{-}Glu]^-$	271.1	40	98
E2−17G	$[M-H]^-$	447.2	$[M\text{-}H\text{-}Glu]^-$	271.0	31	147
F–G	$[M-H]^-$	537.2	$[M\text{-}H\text{-}CH_2OGlu]^-$	331.1	35	105
PD-G	$[M-H]^-$	495.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	36	158
Preg-G	$[M-H]^-$	491.1	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	29	120
THDOC-3G	$[M-H]^-$	509.3	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	40	125
d_3T-G	$[M-H]^-$	466.2	$[M\text{-}H\text{-}Glu]^-$	290.1	30	110
d_4An-G	$[M-H]^-$	469.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2\text{-}CO]^-$	85.0	25	126
d_3E2−3G	$[M-H]^-$	450.2	$[M\text{-}H\text{-}Glu]^-$	274.0	44	127
d_3DHT-G	$[M-H]^-$	468.2	$[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$	113.0	29	121

as glucuronides.

Other steroid glucuronides such as DHT-G, epiAn-G, E1-G, E2−17G, E3−16G, THDOC-3G, Preg-G, epiT-G and PD-G were also measured in our panel. The concentrations of PD-G increased with age up to 687.8 nmol/L in 15 to 20-year-old adolescents. The levels of the other analytes in most of samples were below LOQ.

4. Discussion

4.1. LC–MS/MS method development

Our sample workup, which included protein precipitation and SPE, successfully removed most endogenous interferences from samples. Particularly protein precipitation proved beneficial, since it could be observed in all our samples. While SPE presents a common approach, protein precipitation has often been skipped regarding hitherto published methods on steroid glucuronide analysis [2,15,16,18]. Furthermore, the lifetime of an analytical column will be prolonged when protein clogging is avoided.

In view of the complex composition of urine, an efficient LC method should be capable of differentiating and quantifying various analytes. An-G, DHT-G, epiAn-G and Etio-G are all structural isomers (Fig. 3). They share the same mass transition of *m/z* 465→113. Similarly, T-G, epiT-G and DHEA-G also have the same mass transition *m/z* 463→113. E2−3 G and E2−17 G are both glucuronide conjugates of E2 at position 3 and position 17, respectively. Therefore, good chromatographic separation for each compound is required. Jäntti used two C18 columns installed in series to separate analytes [22]. UHPLC was employed for some studies [15,16]. UHPLC enables analysis on long columns with smaller particle size allowing for better resolution compared with normal HPLC columns. However, the cost of this relatively new technique limits its application in the research laboratories.

In our study, several HPLC columns were tried. C18, Accucore Phenyl -X, Accucore Aq as well as Accucore polar premium from Thermo Fisher scientific were employed, but none of them was successful to get enough separation between Etio-G and An-G. The tiny structural difference between Etio-G and An-G refers to the position of the hydrogen at C5: either α or β. We finally achieved separation of these two compounds on the C30 stationary phase of an Acclaim™ C30 column, which is based on a covalent modification of C30 alkyl silane onto silica gel. This column features high selectivity and separation of structurally related isomers. Furthermore, increasing the percentage of ACN in the mobile phase contributed to successful resolution.

Steroid glucuronides can be detected both in positive or negative mode. Fabregat et al. have shown that in positive mode $[M+H]^+$ was the major ion for steroid glucuronides with a conjugated 3-keto group, while steroids possessing a 17-keto or unconjugated 3-keto group exhibited $[M + NH_4]^+$ as the major ion [30]. The reasons why negative mode was chosen in our study were that a) ammonium hydroxide solution was added to mobile phase to separate analytes and that b) ammonium hydroxide was also beneficial for promoting the ionization in negative mode. The common precursor ion for all steroid glucuronides in negative mode is the deprotonated molecular ion: $[M-H]^-$.

In androgen glucuronides (An-G, epiAn-G, Etio-G, DHEA-G, DHT-G, T-G and epiT-G), Preg-G, PD-G and THDOC-3 G, *m/z* 113 present the common fragment. It originates from the glucuronide moiety. Fig. 4 illustrates the MS fragmentation pathway in negative mode [31]. The glucuronide moiety (*m/z* 175) is cleaved from the deprotonated steroid glucuronide molecule $[M-H]^-$. $[Glu\text{-}H\text{-}H_2O\text{-}CO_2]^-$ (*m/z* 113) is then generated by the loss of H_2O and CO_2.

In estrogen glucuronides, $[M-H\text{-}Glu]^-$ resembles the highest fragment ion. The reason is that the aromatic ring stabilizes the perhydrocyclopentanophenanthrene skeleton. Thus, the glucuronide moiety can break off more easily.

Regarding F–G, the major fragment ion is also $[M-H\text{-}CH_2OGlu]^-$. In this case, glucuronic acid is attached to the 21-hydroxyl group of the side chain of the 4-ring skeleton which obviously is less stable than glucuronidation at 3-hydroxyl group of steroid.

4.2. Free, sulfated and glucuronidated steroids in urine

Glucuronidation occurs mainly in the liver. Biosynthesis of steroid glucuronides (Fig. 5) is catalyzed by uridine diphosphoglucuronosyl transferase (UGTs) enzymes which transfer the glucuronyl group of uridine 5′-diphosphoglucuronic acid (UDPGA) to steroids. UDPGA is biosynthesized from UDP-glucose and NAD + by UDPG dehydrogenase. The production of UDP-glucose requires glucose-1-phosphate and uridine triphosphate (UTP) and is catalyzed by phosphorylase [32].

Steroids can be present in their free or conjugated (i.e. sulfated or glucuronidated) form. Hardly any data exist revealing the distribution of these various forms. To elucidate their distribution in urine, we measured their free, sulfated or glucuronidated forms by LC–MS/MS in children of various age groups and compared these results in an orthogonal approach with their total concentrations after enzymatic hydrolysis by GC–MS. It can be seen from Table 5 that the levels of the free fractions were by far lower than those of the corresponding conjugated fractions. In general, the sums of the glucuronidated, sulfated and free forms of an analyte showed good correspondence with its total

Table 4
Validation parameters of the method.

Steroids	E3−16G	E2−3G	E2−17G	E1-G	FG	TG	DHEAG	epiAnG	DHTG	epiTG	THDOC-3G	PregG	AnG	EtioG	PDG
IS	d_3E_2-3G	d_3E_2-3G	d_3E_2-3G	d_3E_2-3G	d_4AnG	d_3TG	d_3TG	d_4AnG	d_3DHTG	d_3TG	d_4AnG	d_4AnG	d_4AnG	d_4AnG	d_4AnG
Linearity (nmol/L)	4.3−108	4.5−111	5.6−111	2.2−112	1.9−186	5.4−108	5.4−431	5.4−1072	5.4−107	5.4−108	4.9−98	20.3−1015	21.4−10716	10.7−10716	4.0−1007
R^2	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99
LOD (nmol/L)	2.2	2.2	3.3	1.1	0.9	2.2	2.2	2.1	2.1	2.2	2.0	10.2	10.7	5.4	2.0
LOQ (nmol/L)	4.3	4.5	5.6	2.2	1.9	5.4	5.4	5.4	5.4	5.4	4.9	20.3	21.4	10.7	4.0
LOQ Precision (CV%)	13.6	13.0	9.0	7.0	17.1	13.8	7.5	13.1	9.6	7.5	18.4	7.4	9.1	9.4	18.8
LOQ Accuracy (RE%)	89.7	107.3	101.4	81.1	102.6	94.8	88.2	81.2	92.1	87.6	96.8	82.4	88.2	87.2	88.4
Matrix effect	0.97	0.93	1.02	1.03	0.96	1.07	1.07	1.02	0.96	0.87	0.83	0.94	0.97	1.02	0.98
Process Efficiency (%)															
QC 1	108.3	86.5	105.5	110.4	106.1	115.1	99.4	91.4	108.3	83.6	80.7	91.1	92.2	116.1	97.2
QC 2	108.8	89.3	102.4	104.6	99.4	106.5	111.8	100.8	100.9	88.4	79.5	95.7	103.5	106.4	98.8
QC 3	108.3	86.1	100.7	106.3	96.7	103.9	105.3	104.7	98.3	84.1	74.9	94.3	105.2	99.2	96.5
Intra-day precision (CV%, n = 5)															
QC 1	4.6	8.5	9.0	13.7	13.5	14.7	8.7	8.8	10.7	4.8	2.2	8.5	9.7	8.2	3.4
QC 2	8.1	10.6	8.2	5.4	6.5	7.3	6.5	6.4	8.2	5.5	3.7	6.7	3.8	5.6	9.5
QC 3	8.7	6.2	5.9	3.8	6.2	5.0	4.2	3.5	6.8	5.0	5.9	4.5	3.8	5.5	8.5
Intra-day accuracy (RE%, n = 5)															
QC 1	88.0 ± 3.4	88.8 ± 3.7	103.2 ± 6.2	97.8 ± 11.6	95.3 ± 8.6	99.2 ± 6.0	94.9 ± 8.3	87.5 ± 3.7	94.2 ± 9.4	88.4 ± 3.2	96.3 ± 2.1	102.7 ± 8.8	94.1 ± 7.3	88.8 ± 3.3	105.6 ± 8.0
QC 2	112.7 ± 2.9	94.3 ± 6.7	93.5 ± 4.9	98.9 ± 5.3	97.5 ± 8.2	92.4 ± 5.0	95.1 ± 3.1	97.0 ± 5.4	93.6 ± 4.0	89.7 ± 1.7	86.1 ± 3.2	87.8 ± 4.9	99.8 ± 3.2	97.6 ± 2.6	104.2 ± 5.6
QC 3	111.6 ± 3.8	90.0 ± 3.0	92.7 ± 3.3	98.7 ± 7.7	97.2 ± 7.9	95.6 ± 3.7	97.0 ± 2.8	95.4 ± 3.2	98.8 ± 5.1	92.3 ± 4.6	88.2 ± 5.2	87.5 ± 2.3	101.3 ± 3.8	97.7 ± 4.1	92.2 ± 6.1
Inter-day precision (CV%, n = 5)															
QC 1	10.6	10.5	5.6	9.6	8.6	7.8	11.9	11.4	8.3	12.0	9.0	12.1	9.3	12.7	2.3
QC 2	10.9	7.5	3.7	4.2	5.5	4.4	6.4	8.4	6.3	6.9	7.9	9.1	1.6	1.5	6.8
QC 3	3.5	9.9	3.9	6.7	8.1	4.0	6.0	4.2	9.7	8.2	5.9	8.3	1.0	6.9	5.2
Inter-day accuracy (RE%, n = 5)															
QC 1	102.4 ± 10.8	89.5 ± 4.4	96.1 ± 5.4	89.7 ± 3.4	94.2 ± 3.3	100.0 ± 7.8	93.0 ± 7.5	87.3 ± 5.4	92.1 ± 6.7	102.1 ± 12.3	95.6 ± 8.6	113.2 ± 3.7	106.9 ± 8.0	99.7 ± 12.6	103.4 ± 2.4
QC 2	104.2 ± 7.4	97.0 ± 7.3	106.6 ± 8.6	94.9 ± 3.9	97.4 ± 5.4	102.5 ± 4.8	92.9 ± 6.0	90.7 ± 7.7	97.0 ± 6.1	101.2 ± 7.0	91.5 ± 7.2	101.2 ± 9.2	99.8 ± 1.6	99.0 ± 1.4	94.3 ± 6.4
QC 3	102.1 ± 3.5	93.3 ± 9.2	106.0 ± 4.7	97.3 ± 6.5	100.0 ± 8.1	99.1 ± 3.9	94.2 ± 5.7	96.4 ± 3.7	87.8 ± 5.8	97.3 ± 8.0	87.8 ± 2.2	96.8 ± 8.1	100.7 ± 1.0	93.6 ± 6.4	94.7 ± 4.9
Recovery (%)															
QC 1	111.6	93.0	103.4	107.2	110.5	107.6	92.9	89.6	112.8	96.1	97.2	96.9	95.1	113.8	99.2
QC 2	112.2	96.0	100.4	101.6	103.5	99.5	104.5	98.8	105.1	101.6	95.8	101.8	106.7	104.3	100.8
QC 3	111.6	92.6	98.7	103.2	100.7	97.1	98.4	102.6	102.4	96.7	90.3	100.3	108.5	97.3	98.5
QCs (nmol/L)															
QC 1	11	11	11	4	4	11	11	11	11	11	10	41	43	21	11
QC 2	43	45	45	45	93	43	172	429	43	43	39	406	4286	4286	403
QC 3	86	89	89	90	148	86	344	857	86	86	78	812	8572	8572	805

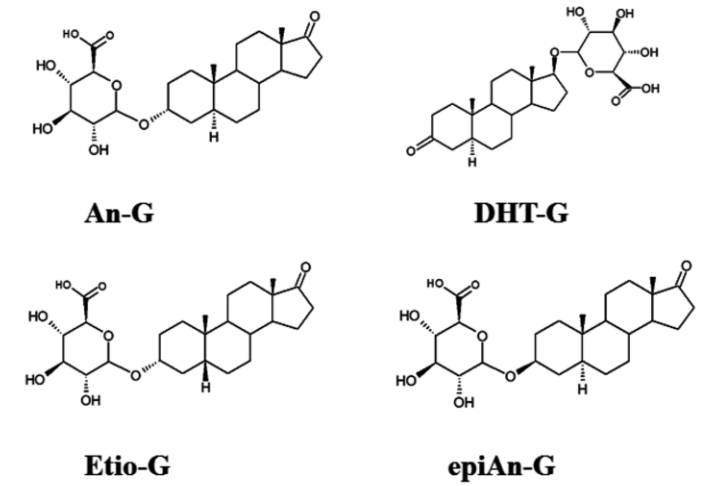

Fig. 3. Structural isomers of androsterone glucuronide (An-G), dihydrotestosterone glucuronide (DHT-G), etiocholanolone glucuronide (Etio-G) and epiandrosterone glucuronide (epiAn-G).

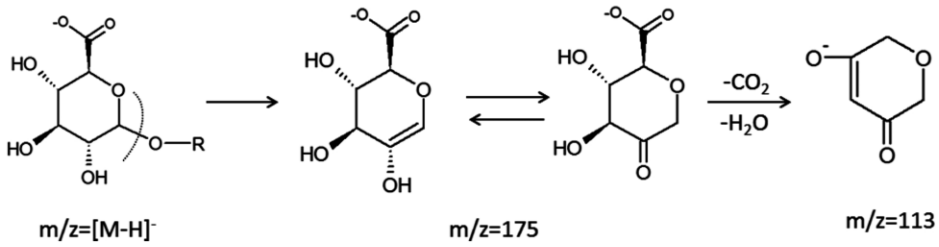

Fig. 4. Main MS fragmentation pathway for steroid glucuronides in negative mode according to [31].

amount determined after enzymatic hydrolysis by GC–MS.

DHEA as well as DHEAS are produced in the zona reticularis of the adrenals. DHEA is sulfated to DHEAS by sulfotransferase (SULT2A1). Thus, DHEA is mostly present in its sulfated form. In infancy, the high levels of DHEA (mean ± SD: 209.6 ± 342.8 nmol/L) were found in one-month-old neonates almost entirely in the sulfated form (184.5 ± 314.0 nmol/L). The reason is that the fetal zone of the adrenal produces tremendous amounts of DHEAS [33]. DHEA declined to its lowest levels in children aged 1−12 months. This reflects involution of the fetal zone taking place in the first months of life [34]. Thereafter, the concentrations of DHEA continued to rise again up to the age of 20 years. Adrenarche refers to the rise of adrenal androgens produced in the developing zona reticularis between 6 and 8 years of age [33]. The onset of increased urinary excretion of DHEA we observed suggested that the phenomenon of adrenarche is also reflected in urine.

Androsterone (An) and etiocholanolone (Etio) are metabolites of DHEA, androstenedione and testosterone [4]. They present two dominant androgen metabolites in urine of children and adolescents [35,36]. The GC–MS data showed that the levels of An and Etio increased substantially in children during childhood. Our results are in accordance with Remer et al. [37] who have shown that DHEA, An as well as Etio increased clearly after the age of 3 years and provide further evidence that adrenache is a gradual process starting much earlier than hitherto believed. We found that An and Etio were mostly excreted as sulfates and glucuronides into urine at all age groups. It had recently been shown that sulfated steroids can be transported into cells by specific carriers where then the sulfate group can be cleaved to modulate the inner cell milieu [38]. In the group of 1−5 year-old children, sulfated and glucuronidated forms contributed equally to the total amounts of An and Etio, respectively. Our data showed that glucuronidation is preferred over sulfation in older children compared to younger children.

Cortisol is secreted by the zona fasciculata of the adrenal glands [39]. Urinary concentrations of total cortisol measured by GC–MS were found to be relatively constant during the first 20 years of age. This finding is in accordance with previous results [36]. In our study we analyzed cortisol 21-sulfate and cortisol 21-glucuronide by LC–MS/MS. The good correspondence between total cortisol measured by GC–MS and the sum of free, sulfated and glucuronidated cortisol measured by LC–MS/MS indicates that the 21-hydroxy group of cortisol is the favored position for conjugation. The hydroxyl groups at positions 11 and 17 of cortisol are obviously sterically hindered. Interestingly, cortisol was found to be present almost entirely in its sulfated form in urine of newborns. Its sulfated portion still accounted for one-third of the total cortisol excretion in urine of children up to the age of 15−20 years (Table 5). It is highly likely that cortisol, as a major secretory product of the adrenals, is sulfated in the adrenals [40]. While the fraction of cortisol sulfate decreased with age, the fraction of its glucuronide increased. In contrast, the concentrations of unconjugated cortisol remained rather constant throughout childhood. In the two older age groups (Table 5), each form contributed approximately equally to the total amount of cortisol.

Testosterone was found to be excreted mostly in its glucuronidated form [41]. Testosterone glucuronide was found approximately 2–4 times

Table 5
Concentrations of free, sulfated and glucuronidated steroids in urine (nmol/L).

Age group	Item	An				Etio				DHEA				F				T				E2			
		LC-MS/MS			GC-MS	LC-MS/MS			GC-MS	LC-MS/MS			GC-MS	LC-MS/MS			GC-MS	LC-MS/MS			GC-MS	LC-MS/MS			GC-MS
		F	S	G	Total	F	S	G	Total	F	S	G	Total	F	S	G	Total	F	S	G	Total	F	S	G	Total
0–1 month (n = 12)	Median	0.0	0.0	27.9	53.7	0.0	0.0	0.0	0.0	54.7	43.2	0.0	68.7	2.9	182.0	0.0	142.4	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Min	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.3	0.0	0.0	1.5	92.5	0.0	89.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Max	0.0	0.0	234.9	225.7	0.0	0.0	31.9	51.1	169.9	1114.6	37.8	1237.4	16.7	238.0	10.1	300.1	14.0	0.0	50.3	61.9	0.0	0.0	0.0	0.0
1–12 month (n = 12)	Median	0.0	0.0	20.4	53.3	0.0	0.0	0.0	9.0	0.0	4.0	0.0	0.0	12.4	79.6	14.4	93.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Min	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	3.1	41.5	0.0	59.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Max	0.0	13.2	227.6	298.2	0.0	13.9	66.4	40.7	18.0	27.1	21.5	57.7	216.5	168.8	44.1	256.1	10.2	1.9	0.0	0.0	0.0	0.0	0.0	11.2
1–5 year (n = 10)	Median	0.0	32.4	31.4	105.2	0.0	16.8	26.7	96.2	0.0	4.7	0.0	0.0	24.8	59.8	17.8	76.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Min	0.0	0.0	0.0	36.3	0.0	0.2	0.0	29.9	0.0	0.0	0.0	0.0	1.5	19.6	3.4	32.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Max	39.5	1067.8	1114.8	1700.1	58.3	335.1	462.1	688.2	26.8	1240.4	68.0	1559.9	118.4	127.0	113.3	830.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5–10 year (n = 12)	Median	0.0	635.4	451.0	797.2	4.9	214.4	566.0	785.8	0.0	103.6	35.8	137.1	38.6	75.8	27.2	95.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Min	0.0	5.1	174.2	360.9	0.0	71.2	172.5	244.6	0.0	13.3	0.0	55.1	17.8	29.7	18.2	71.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Max	28.6	1155.1	1620.1	2864.2	27.9	1391.2	2439.2	2746.3	16.2	2651.4	82.3	3406.2	68.2	114.6	60.2	218.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Min	10–15 year (n = 11)	Median	0.0	553.7	1115.5	1655.7	6.4	309.2	1273.9	1302.1	0.0	102.3	50.1	133.9	29.0	33.3	26.0	101.1	0.0	1.9	8.5	0.0	0.0	0.5	5.7	0.0
		0.0	77.3	362.2	632.9	3.3	34.2	208.7	240.4	0.0	16.0	6.5	63.6	2.7	9.8	7.7	55.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Max		2.6	2497.3	8297.9	10673.7	21.1	1011.4	3884.7	3721.4	34.8	7192.5	278.3	8125.6	138.1	63.5	124.9	260.8	0.8	27.6	53.0	60.3	0.0	0.9	17.7	29.2
15–20 year (n = 10)	Median	2.4	1171.2	4368.9	5994.3	12.5	408.4	3681.9	3783.0	0.0	297.4	160.2	385.3	46.6	38.6	50.0	120.1	0.0	8.1	0.0	0.0	0.0	0.4	9.4	10.5
	Min	0.0	52.7	957.2	935.1	3.3	35.9	627.3	540.7	0.0	117.0	16.5	89.6	17.9	14.9	12.4	40.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Max	126.8	3787.2	9485.0	11061.0	161.4	3221.4	7839.5	9330.1	74.3	7744.4	202.6	11890.5	59.3	57.3	131.2	167.8	6.2	38.2	144.7	133.3	0.0	2.3	18.4	24.9

F, S and G stand for free, sulfate and glucuronide fractions measured by LC–MS/MS, respectively. Total concentrations of steroids were measured after enzymatic hydrolysis by GC–MS.

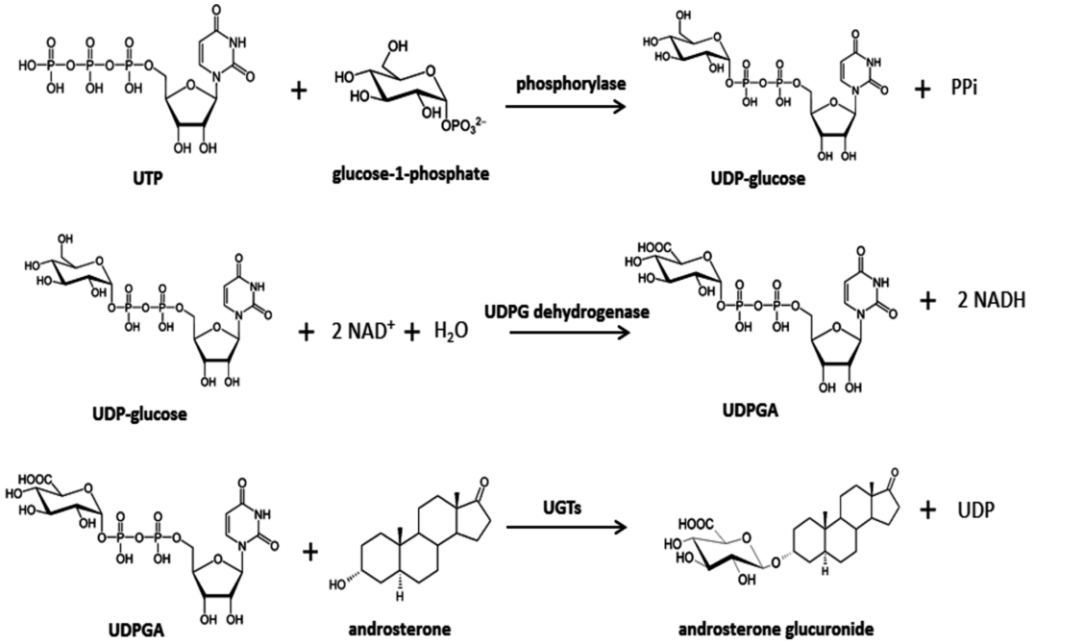

Fig. 5. Reaction mechanism of glucuronidation (UTP, uridine triphosphate; PPi, pyrophosphate; UDP-glucose, uridine diphosphate glucose; NAD+/NADH, oxidized/reduced nicotinamide adenine dinucleotide; UDPGA, uridine 5′-diphosphoglucuronic acid; UDP, uridine diphosphate).

higher than testosterone sulfate. Only a very little amount of unconjugated testosterone was found in older children. The only sex difference was found in the group of 15−20 year-old children for total testosterone (female: 9.2 ± 22.6 nmol/L; male: 83.3 ± 58.7 nmol/L; $P < 0.05$). Theoretically, estradiol can be sulfated or glucuronidated at either C-3 or C-17. The concentrations of E2−17S and E2-17G were both below LOQ. However, the good correspondence between E2−3G measured by LC–MS/MS and total estradiol measured by GC–MS suggested that estradiol was predominantly glucuronidated at position 3 in urine samples.

The number of our analytes was limited by the commercial unavailability of other reference steroid glucuronides. Furthermore, also stable isotope labeled internal standards were only available in a limited number. As further research into the metabolism and analysis of steroid glucuronides might widen our understanding of their biological roles, greater commercial availability of such reference standards would be desirable.

In conclusion, we have developed and validated a LC–MS/MS method for quantification of steroid glucuronides in human urine. The method was applied to urinary samples of children and adolescents to characterize the urinary excretion of conjugated and unconjugated steroid metabolites. Clinical routine steroid hormone diagnostics is hitherto focused on the assessment of unconjugated steroid hormones. Our study shows that conjugated forms of steroid hormones, both sulfates as well as glucuronides, are present in significant amounts in biological fluids, e.g. urine. Little is known about the biological role of steroid hormone conjugates. Furthermore, these steroid hormone conjugates might bear potential as future biomarkers in steroid hormone related diseases.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2020.105774.

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3 Results and discussion

3.1 Analyzing free and sulfated steroids in amniotic fluid of mid-gestation by LC-MS/MS

Amniotic fluids (AF), the fluid surrounding the fetus, is swallowed and excreted by the fetus. The levels of steroids in AF are essential for the development of fetus. To study the free and sulfated steroids in AF, LC-MS/MS was employed. Based on literature research, only one paper has so far used LC-MS/MS to measure eleven steroids and one sulfated steroid-DHEAS (Fahlbusch et al., 2015). To the best of our knowledge, the levels of other sulfated steroids have not been reported previously.

Quantifying multiple steroids simultaneously is complicated due to the complex nature of amniotic fluid and wide concentration ranges of steroids. There are isobaric compounds and structural isomers coexisting in the samples. Thus, protein precipitation and SPE were used to obtain clean extracts of targeted analytes. Stable isotope labeled internal standards were added to the samples for correcting the loss of analytes during sample analysis.

Free steroids were extracted by chloroform using SPE. In a preliminary study, we found that estrogens were present in very low concentrations in AF. Therefore, derivatization was introduced to improve the sensitivity of estrogens (Figure 16). Dansyl chloride was used as the derivatization agent. Sodium bicarbonate buffer, derivatization time and reconstitution solution were optimized to ensure maximal derivatization, which also improve the sensitivity of estrogens from 2.5 ng/mL to 0.2 ng/mL. Different analytical columns and mobile phase combinations were tested for the best chromatographic separation of analytes and interfering compounds. The Accucore Polar Premium column was chosen for the best resolution between analytes and other interferences. APCI in positive mode was used to detect free steroids, because steroids with a 3-keto-4-ene structure enabled them to easily capture protons from aqueous solution to generate positive ions. Different mass spectrometric parameters including vaporizer temperature, capillary temperature, sheath gas, auxiliary gas and discharge current were evaluated for the maximal ionization.

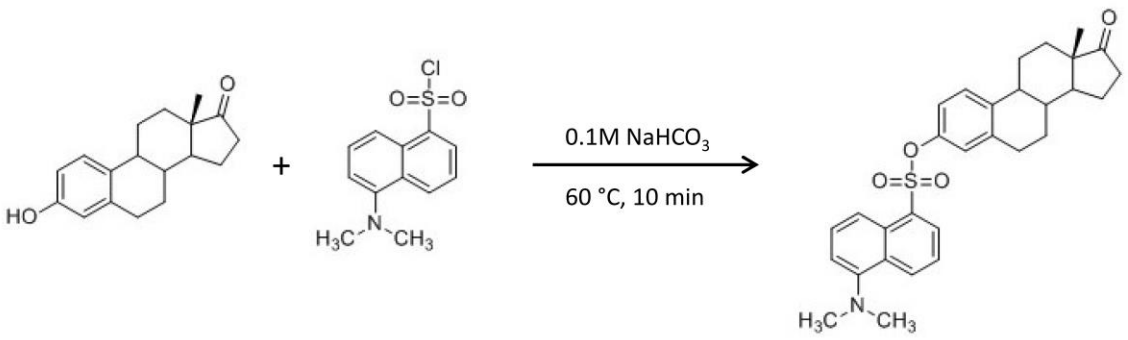

Figure 16. The derivatization reaction between E1 and dansyl chloride was carried out at 60°C for 10min in 0.1M NaHCO3 buffer.

Sulfated steroids were extracted by methanol using SPE. 14 sulfated steroids including estrogen sulfates, androgen sulfates and progestogen sulfates were measured in the extracts. Sulfated steroids are more polar due to the introduction of sulfate group in the structure. They appear in solution in the form of anions. Hence, ESI in negative mode was employed to measure sulfated steroids. MS conditions were optimized to obtain the best signals. Several structurally similar compounds sharing the same mass transition increased the complexity of measurement. For example, testosterone sulfate (retention time, RT: 3.90 min), epitestosterone sulfate (RT: 4.30 min) and DHEAS (RT: 5.56 min) are isomers sharing the same MRM transition (m/z: 367→97); androstenediol sulfate (RT: 3.80 min), dihydrotestosterone sulfate (RT: 5.74 min), epiandrosterone sulfate (RT: 6.17 min) and androsterone sulfate (RT: 6.82 min) also have the same mass transition (m/z: 369→97) (Figure 17). In this case, baseline separation of these analytes on chromatography is of importance for reliable results.

The method requires 600 µL of AF. After the method was developed, validation was conducted to ensure the performance of the method following the guidelines of Food and Drug Administration (FDA) for bioanalytical method validation. Good linearity with R>0.99 in the calibration range was found for all analytes. The recovery of the method was within 100% ± 15%. Accuracy, precision and recovery were evaluated at 3 quality control (QC) levels which were defined according to the physiological concentrations. Intra-day/inter-day accuracy and precision were all below 15% for the QCs. There was no significant matrix effect for all analytes.

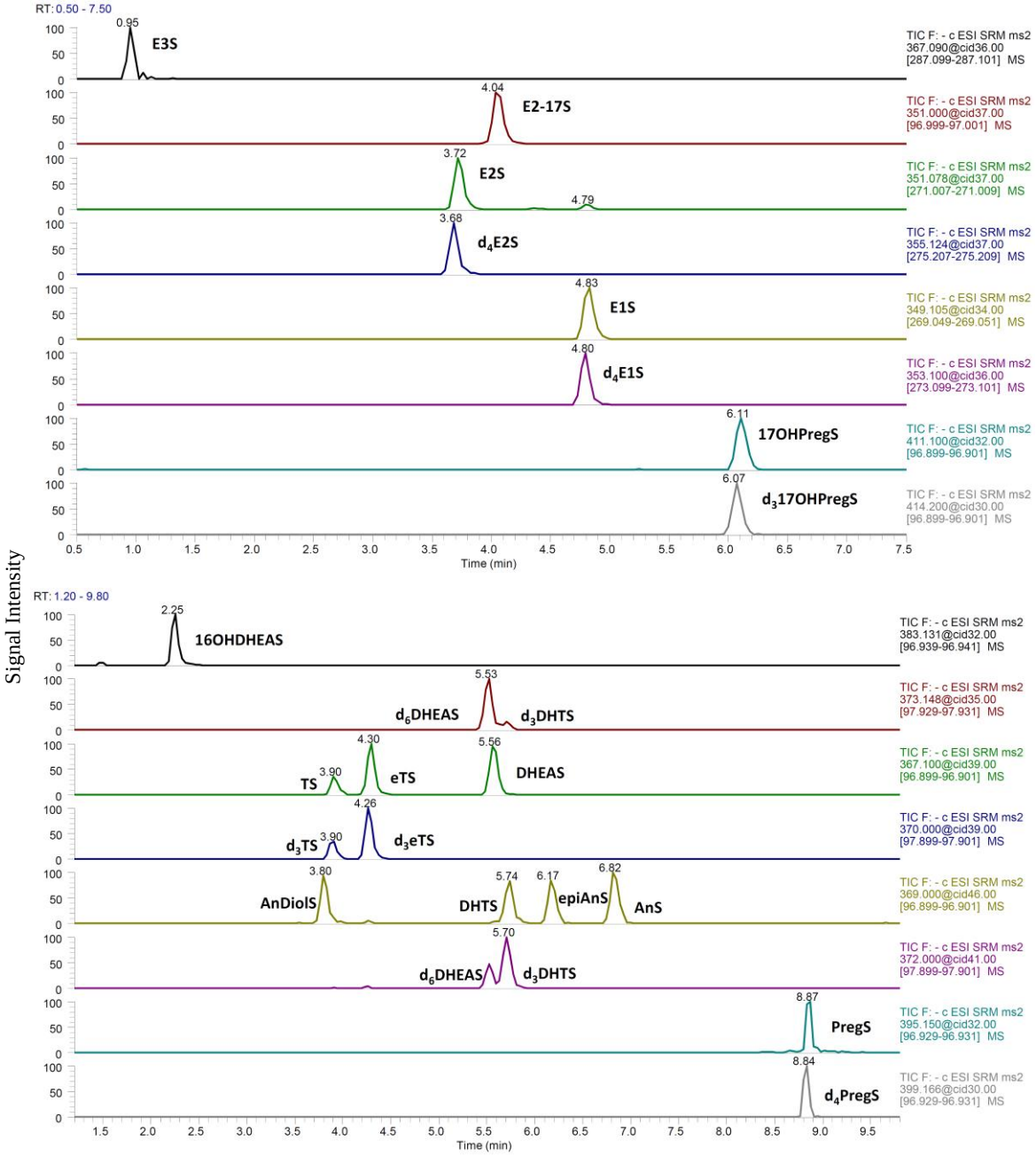

Figure 17. Chromatogram of spiked sulfated steroid standards in stripped urine (10 ng/ml; all targeted analytes were chromatographically separated; E3S, estriol 3-sulfate; E2-17S, estradiol 17-sulfate; E2S, estradiol 3-sulfate; E1S, estrone sulfate; 17OHPregS, 17α-hydroxypregnenolone sulfate; 16OH-DHEAS, 16α-hydroxydehydroepiandrosterone sulfate; TS, testosterone sulfate; eTS, epitestosterone sulfate; DHEAS, dehydroepiandrosterone sulfate; AnDiolS, 3β,17β-androstenediol 3-sulfate; DHTS, dihydrotestosterone sulfate; epiAnS, epiandrosterone sulfate; AnS, androsterone sulfate; PregS, pregnenolone sulfate.).

The new LC-MS/MS method was then applied to 65 human AF samples of mid-gestation. 14 sulfated and 6 unconjugated steroids were measured, of which, 13 sulfated and 3 unconjugated steroids were measured for the first time in AF of mid-gestation. Progesterone (mean±SD: 37.4±12.8 ng/mL) presented the highest concentration of all steroids measured. The highest level of sulfated steroids was found for 16OH-DHEAS (mean±SD: 21.5±10.7 ng/mL). Only testosterone showed a significant sex difference ($p<0.0001$). This is the first paper presenting comprehensive reference data on free and sulfated steroids in AF of mid-gestation.

Moreover, strong positive correlations were found between 16OH-DHEAS and DHEAS, 16OH-DHEAS and estriol sulfate (E3S), E3 and E3S, 17OH-pregnenolone sulfate (17OHPregS) and pregnenolone sulfate (PregS). These findings support the concept of the classical steroid pathway in the feto-placental unit (Ng, 2000). Large amounts of DHEAS are produced by the fetal adrenal glands. Then DHEAS is hydroxylated at position 16α in the fetal liver to yield 16OH-DHEAS. Thereafter, 16OH-DHEAS is metabolized to estriol (E3) by placental sulfatase, 3β-HSD, 17β-HSD and aromatase. Afterwards, E3 is sulfated by sulfotransferase to produce E3S (Geyer et al., 2017). PregS and 17OHPregS also showed a very good correlation between each other in AF. It has been demonstrated that PregS can be biosynthesized to 17OHPregS in vitro and in the human male (Neunzig et al., 2014) (Sánchez et al., 2016). This proposed sulfated steroid pathway is also likely to be present in the feto-placental unit.

Reference data of 14 sulfated and 6 unconjugated steroids in amniotic fluids were obtained for future prenatal diagnosis studies of congenital adrenal hyperplasia and low-estriol diseases such as X-linked steroid sulfatase deficiency, aromatase deficiencies, oxidoreductase deficiencies or Smith-Lemli-Opitz Syndrome.

3.2 Quantitative targe ted GC-MS based steroid metabolome analysis of amniotic fluid of mid-gestation

To complement our findings of LC–MS/MS study (Wang et al., 2019), we used GC-MS to measure further steroid hormone metabolites in AF by a comprehensive steroid hormone metabolomics analysis approach. LC-MS/MS with soft ionization techniques such as electrospray ionization or atmospheric pressure chemical ionization is not suitable for analyzing steroids bearing a 3β-hydroxy-5-ene or saturated ring structure. However, electron impact ionization used with GC-MS overcomes the ionization problem. Thus, GC-MS is more suitable for providing an integrated picture of the steroid metabolome. Furthermore, GC-MS has the advantage of much higher separation power and sensitivity. Consecutively, GC-MS has proved to be a powerful tool for the delineation of steroid metabolomes and the diagnosis of clinical disorders (Krone et al., 2010) (Kulle et al., 2017). Therefore, GC-MS was employed to further analyze a multitude of steroids in AF of mid-gestation.

However, in contrast to LC-MS/MS, GC-MS does not allow for the determination of intact steroid conjugates (Shackleton et al., 2018). Therefore, hydrolysis of conjugates is required to remove the conjugate moiety, i.e. sulfate or glucuronide. For this reason, the concentrations reported in the present study represent total concentrations of free and conjugated steroids. The concentrations or excretion rates of metabolites obatined from GC-MS analysis are usually compared with reference values for the diagnosis of steroid disorders. Ratios of precursor metabolites to product metabolites can be used to assess enzyme activities (Wudy et al., 2018).

A targeted GC-MS analysis method was used to quantify 52 steroids in AF. Fetal and neonatal steroid metabolites are always hydroxylated at two or more positions, e.g. 1β, 6α/β, 15β, 16α/β and form steroid conjugates (Hill et al., 2010). Firstly, free and conjugated steroids were extracted by SPE from 5 ml of AF. Afterwards, hydrolysis of the conjugated steroids by type H-1 sulfatase from helix pomatia was carried out. Type H-1 sulfatase from helix pomatia has not only sulfatase activity but also β-glucuronidase activity. The hydrolyzed steroids were recovered by a second SPE step. Then derivatization of steroids was done to form methyloxime-trimethylsilyl ethers (Wudy et al., 2018).

After derivatization, steroids were volatile and stable under high temperature in the GC chamber. GC-MS operating in SIM mode was used for monitoring all steroids. In total, 52 steroids including pregnenolone and 17-OH-pregnenolone metabolites, dehydroepiandrosterone (DHEA) and its metabolites, progesterone and 17-OH-progesterone metabolites, sex hormones as well as corticosterone and cortisol metabolites were measured by the method (Table 1a and 1b in Publication 2). Figure 18 depicts the representative chromatograms for typical steroid metabolites of each group.

The highest concentrations of all groups were found for the sum of pregnenolone and 17-OH-pregnenolone metabolites. 5-pregnene-3β,20α,21-triol (P5-3β,20α,21-triol) (mean ± SD: 45.3 ± 23.2 ng/mL) and 5-pregnene-3β,21-diol-20-one (21-OH-P5olon) (43.9 ± 24.0 ng/mL) showed the highest levels within this group.

16α-OH-DHEA (42.4 ± 21.8 ng/mL) and 16β-OH-DHEA (32.9 ± 30.3 ng/mL) were the two dominant steroids in the group of DHEA and its metabolites. In our previous paper (Wang et al., 2019), a strong correlation between DHEAS and 16α-OH-DHEAS was found in AF by LC-MS/MS. However, no correlation was found for total DHEA and total 16α-OH-DHEA as well as total DHEA and total 16β-OH-DHEA in the present study. We speculated that DHEA might not be the only precursor of 16α-OH-DHEA and 16β-OH-DHEA in the feto-placental unit. The strong correlation between 16α-OH-P5olon and 16α-OH-DHEA (r=0.83), 16α-OH-P5olon and 16β-OH-DHEA (r=0.76), respectively, indicated that 16α-OH-P5olon is likely main precursor of 16α-OH-DHEA and 16β-OH-DHEA. Following the Δ5 pathway, 16α-OH-P5olon is metabolized to 16α,17α-OH-pregnenolone by 17α-hydroxylase, then it is converted to 16α-OH-DHEA by 17,20 lyase. There was a strong correlation between 16α-OH-DHEA and 16β-OH-DHEA (r = 0.88). The explanations could be that they might be produced from the same precursor or a hydroxysteroid epimerase (HSE) that converts the 16-hydroxy group between α and β positions.

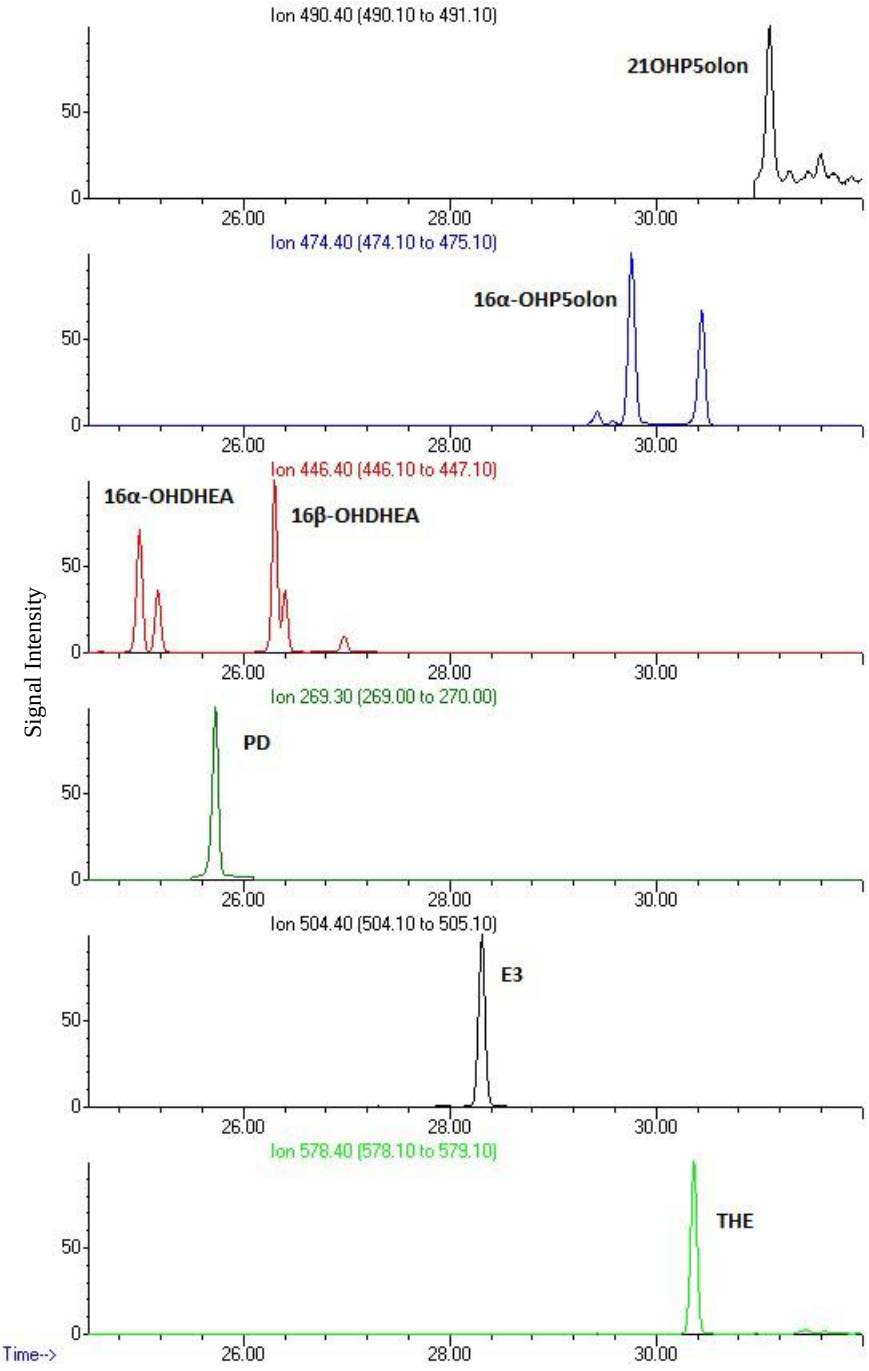

Figure 18. Representative SIM-chromatograms of important steroids such as the pregnenolone metabolites 21-OH-P5olon and 16α-OH-P5olon, the DHEA metabolites 16α-OH-DHEA and 16β-OH-DHEA (both appear as double peaks), the progesterone metabolite pregnanediol (PD), estriol (E3) and the cortisol metabolite tetrahydrocortisone (THE) in an AF sample of mid-gestation. (X: retention time, Y axis: abundance of signal).

In the group of progesterone and 17-OH-progesterone metabolites, the predominant steroid was 5β-pregnane-3α,20α-diol (pregnanediol) (91.6 ± 41.0 ng/mL). In fact, the concentration of pregnanediol was also the highest of all measured individual steroid hormone metabolites in AF samples. Pregnanediol is the main metabolite of progesterone.

The primary estrogen metabolite was E3 (33.2 ± 26.1 ng/mL). In the group of androgens, testosterone showed a highly significant sex difference ($p<0.0001$). Unlike the classic pathway depicted in Figure 3, 17-hydroxy-progesterone is converted to dihydrotestosterone (DHT) through 17α-hydroxy-allopregnanolone in the "backdoor" pathway, which bypasses the conventional intermediates androstenedione and testosterone. The ratio between androsterone (An) and etiocholanolone (Etio) can be used as an indicator for the activity of the alternative "backdoor" pathway. Furthermore, the ratio between 17α-hydroxy-allopregnanolone and 11−OH-androsterone can be used to calculate the activity of "backdoor" pathway vs. that of the Δ4 pathway (Kamrath et al., 2018). In our study, no significant sex differences were found for both ratios ($p=0.71$; $p=0.38$). We conclude that the steroid hormones in AF of mid-gestation were not indicative of the "backdoor" pathway.

The highest concentrations of all measured cortisone and cortisol metabolites were tetrahydrocortisone (THE). The interconversion between cortisone and cortisol is catalyzed by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Kamrath et al., 2014). 11β-HSD2 converts active cortisol to inactive cortisone, whereas 11β-HSD1 converts cortisone to cortisol (Kosicka et al., 2018). We also found the sum of cortisone metabolites was higher than the sum of cortisol metabolites. This illustrated a preference of cortisol inactivation in the feto-placental unit.

We used GC-MS as a tool to delineate steroid hormone metabolites in AF of mid-gestation by a comprehensive targeted steroid analysis approach. In our study, reference data of levels of 52 steroids in AF of mid-gestation were provided.

Normal gestation-specific reference values for steroid hormones in amniotic fluid are now available to facilitate further studies regarding steroid hormone metabolism during pregnancy and lay the foundation for future diagnosis of various disorders of steroid metabolism.

3.3 Targeted LC-MS/MS analysis of steroid glucuronides in human urine

Glucuronidation is a major route for steroids to eliminate from human body. On the other side, steroid glucuronides can be transformed to their active free forms in gut lumen by cleavage of the conjugate (Li et al, 2019). However, hardly any information is available concerning the physiological levels of intact steroid glucuronides in human body fluids, e.g. urine.

So far, only a few groups have investigated intact steroid glucuronides in human urine by MS-based analytical techniques. However, validation data are sometimes missing (Bowers et al., 1996) (Jaentti et al., 2013) (Anizan et al., 2011) or concentrations have not been reported (Ke et al., 2015) (Badoud et al., 2011). Thus, we planned to develop a reliable analytical method for analyzing concentrations of steroid glucuronides.

Initially, reference standards of 15 steroid glucuronides as well as possible corresponding internal standards were purchased from suppliers. Each standard and internal standards (IS) was studied individually for their respective mass transition, collision energy and tube lens offset. Afterwards, mass spectrometric parameters such as spray voltage, vaporizer temperature, capillary temperature, sheath gas and auxiliary gas were studied. The parameters were optimized for best signals.

Due to the complex composition of urine, an efficient LC method should be able to differentiate and quantify various analytes. An-G, DHT-G, epiAn-G and Etio-G are all structural isomers. They share the same mass transition of m/z 465→113. T-G/epiT-G/DHEA-G (463→113) and E2-3G/E2-17G (447→271) are also isobaric compounds. Several HPLC columns were tried to separate these isomers. Accucore polar premium, Accucore aq as well as Accucore phenyl –X columns were tested, but neither of them was capable of separating Etio-G from An-G. The structural difference between Etio-G and An-G is at C5 position: either α-H or β-H. Finally, an AcclaimTM C30 column with C30 stationary phase was successful to separate these two compounds. Increasing ACN percentage in the mobile phase also improved the resolution.

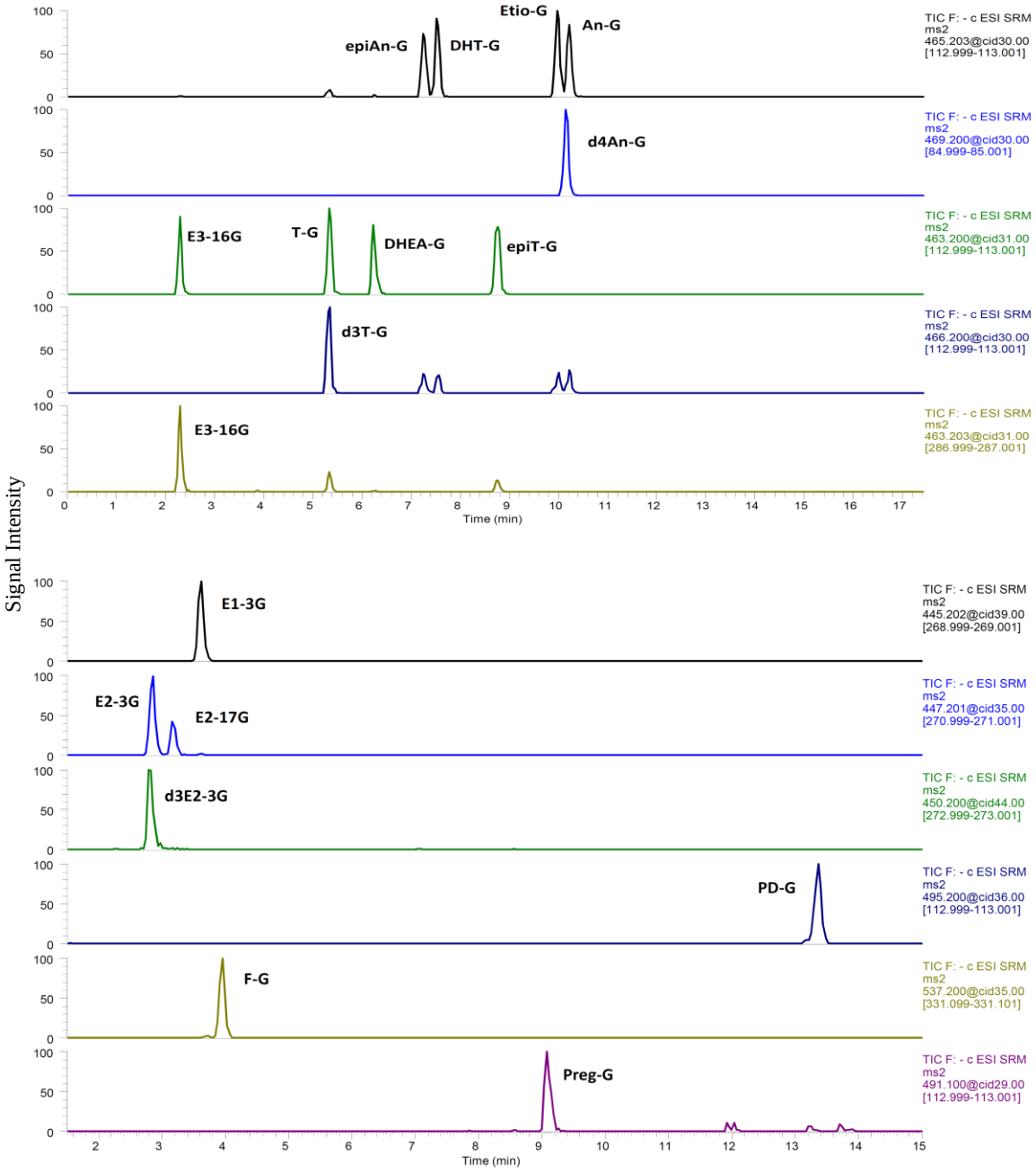

Figure 19. Chromatograms of spiked steroid glucuronide standards (100 ng/mL) quantified by LC-MS/MS. (An-G, androsterone glucuronide; Etio-G, etiocholanolone glucuronide; epiAn-G, epiandrosterone glucuronide; DHT-G; dihydrotestosterone glucuronide, DHEA-G, dehydroepiandrosterone glucuronide; T-G, testosterone glucuronide; epiT-G, epitestosterone glucuronide; E1-3G, estrone glucuronide; E2-17G, 17β-estradiol 17-glucuronide; E2-3G, 17β-estradiol 3-glucuronide; E3-16G, estriol 16-glucuronide; Preg-G, pregnenolone glucuronide; THDOC-3G, tetrahydro-11-deoxycorticosterone 3-glucuronide; F-G, cortisol 21-glucuronide; PD-G, pregnanediol glucuronide.).

A new LC-MS/MS method was developed for determining 15 steroid glucuronides simultaneously. The method requires 2 mL of urine. The chromatograms of steroid glucuronides in steroid-free matrix are depicted in Figure 19. The method was then fully validated according to the requirements of the US Food and Drug Administration (Food and Drug Administration, 2018) . Intra-day and inter-day accuracy (% relative error) and precision (% coefficient of variation) were studied at three different quality control levels. Our method had good accuracy and precision (below 15%). Recovery of all quality controls met the criteria (within 100% ± 15%). No significant matrix effects were observed.

Steroids are present in biological fluids in either free or their conjugated (i.e. sulfated or glucuronidated) forms. The distribution of these various forms of steroids in biological fluids has not been investigated in detail. To explore the proportions of free and conjugated steroids in urine of various age groups, we used LC-MS/MS to determine concentrations of free, sulfated and glucuronidated steroids and then compared these data to their respective total concentrations measured by GC-MS after enzymatic hydrolysis.

DHEA was excreted mostly in its sulfated form. In neonates, the high levels of DHEA (mean ± SD: 209.6 ± 342.8 nmol/L) were found almost entirely as sulfates (184.5 ± 314.0 nmol/L). The reason is that the fetal zone of fetal adrenals produces large amounts of DHEAS. DHEA declined to its lowest concentrations in children aged 1-12 months. This reflects the degeneration of the fetal zone several months after delivery. Then the levels of DHEA continued to increase again until the age of 20 years. The phenomenon of the raise in androgen secretion by the zona reticularis of the adrenal glands between 6 and 8 years of age is called adrenarche. The increase of urinary excretion of DHEA showed that adrenarche is also reflected in urine.

Androsterone and etiocholanolone are metabolites of DHEA, androstenedione and testosterone (Kalogera et al., 2013). The total mean levels of androsterone and etiocholanolone showed an increase up to 5820.0 nmol/L and 4017.8 nmol/L in the group of 15-20-year-old children, respectively. Glucuronide conjugates (4374.3 nmol/L and 3588.5 nmol/L, respectively) dominated. Furthermore, our study proved that glucuronidation is preferred over sulfation in adolescents compared to younger children.

Cortisol is secreted by the zona fasciculata of the adrenal glands (Wudy et al., 2007). Our data showed that cortisol was present primarily in its sulfated form in newborns and its sulfated portion is still account for one-third of total cortisol excretion in the urine of children up to the age of 15-20 years. it is very likely that cortisol, which is mainly secreted by adrenal glands, is also sulfated in adrenal glands, and secreted into circulation. This could be attributed to the abundant steroid sulfotransferase in adrenal glands. Total levels of cortisol were relatively constant with age. While the fraction of cortisol sulfates decreased with age, the fraction of cortisol glucuronides increased with age. Besides, the levels of unconjugated cortisol remained constant throughout childhood.

Testosterone was mainly excreted in its glucuronidated form. Only a very little amount of unconjugated testosterone was found in older children. Our results suggest that estradiol is exclusively glucuronidated at position 3.

The limited availability of the reference standards and stable isotope labeled internal standards of steroid glucuronides restricted our panel of analysis. As further research into the analysis of steroid glucuronides might widen our understanding of their biological roles, it would be desirable that more reference standards are available commercially. Our study shows that conjugated forms of steroid hormones, both sulfates as well as glucuronides, are present in significant amounts in biological fluids, e.g., urine. Moreover, the sums of unconjugated, sulfated and glucuronidated steroids were in good agreement with the respective total concentrations determined after enzymatic hydrolysis by GC-MS.

This work provides unprecedented new insights into age-specificity of children steroid metabolome and gives further information on the free, sulfated, and glucuronidated fractions of urinary steroid hormone excretion. The age-based reference ranges for 52 metabolites will facilitate the interpretation of steroid profiles in clinical research.

4 Conclusion

Traditionally, steroid analysis has relied on immunoassays for a long time (Wudy et al., 2018). Still a lot of steroid measurements are carried out by immunoassays, because of their technical ease and low cost. However, only one analyte can be measured per assay and the specificity of immunoassays is often low. Mass spectrometry-based methods with superior specificity will be the method of choice in steroid hormone measurements in the future. By coupling mass spectrometry with chromatographic technology (LC or GC), the simultaneous determination of multiple steroids can be achieved (metabolomics approach). GC-MS is the most powerful tool for characterizing steroid metabolomes. LC-MS, which is a high throughput technology, is also suitable for analyzing complex steroids, e.g. steroid sulfates or steroid glucuronides. GC–MS and LC–MS are two complementary techniques in steroid analysis.

A new LC-MS/MS method was developed and fully validated for simultaneous quantification of 6 free and 14 sulfated steroids including C18, C19 and C21 steroids in amniotic fluid of mid-gestation. Reference values of these steroids were provided. Most of the steroids have been quantified for the first time in amniotic fluid by LC-MS/MS. The highest concentrations were found for progesterone (37.4±12.8 ng/mL) and16α-hydroxydehydroepiandrosterone sulfate (21.5±10.7 ng/mL). Correlations between AF steroids not only confirmed the classical steroid pathway, but also pointed to a sulfated steroid pathway.

A targeted GC-MS based method was used to further delineate the steroid metabolome of amniotic fluid of mid-gestation. Reference concentrations for a total of 52 pregnenolone and 17α-hydroxypregnenolone metabolites, DHEA and its metabolites, progesterone and 17α-hydroxyprogesterone metabolites, androgens, estrogens as well as cortisone and cortisol metabolites were characterized in amniotic fluid by GC-MS. The dominating steroids were the group of pregnenolone and 17-OH-pregnenolone metabolites (mean ± SD: 138.0 ± 59.3 ng/mL). Cortisol metabolites were clearly present (59.6 ± 13.6 ng/mL) before fetal *de novo* synthesis of cortisol.

A new LC-MS/MS method was developed and validated for quantification of steroid glucuronides in human urine. Steroid hormones are present in biological fluids in unconjugated, sulfated and glucuronidated forms. The remarkable steroid metabolites

were androsterone and etiocholanolone. The total mean levels of them showed an increase up to 5820.0 nmol/L and 4017.8 nmol/L in the group of 15-20 year-old children, respectively. Glucuronide conjugates (4374.3 nmol/L and 3588.5 nmol/L, respectively) dominated. DHEA was excreted mostly as sulfate (0-1 month of age: 184.5 nmol/ L; 15-20 years of age: 1618.4 nmol/L) in all age groups. Cortisol was present predominantly as sulfate (173.8 nmol/L) in newborns.

Steroid conjugates are no longer considered as “passive” excretory products but have biological functions, for example, DHEAS and 16α-OH-DHEAS serve as precursors for placental estrogen biosynthesis. Androgen glucuronides can be deconjugated in gut lumen to free androgens. The application of LC-MS/MS and GC-MS in steroid analysis offers researchers with new perspectives in hormone-dependent malignancies and steroid-studies. Endocrine studies by LC-MS/MS and GC-MS are focused on elucidating biosynthetic pathways and biochemical processes of steroids. Moreover, LC-MS/MS and GC-MS is widely used for the diagnosis of disorders of steroid biosynthesis and metabolism such as Cushing syndrome, Recessive X-Linked Ichthyosis, and congenital adrenal hyperplasia. Therefore, reference data obtained from our studies are very useful for future diagnostic studies of steroid metabolic disorders, in which samples from patients with steroid metabolic disorders should be collected, analyzed, and compared with our reference values. Furthermore, our fully validated LC-MS/MS methods can facilitate transporter studies of unconjugated and conjugated steroids.

5 Summary

In our studies, we used both LC-MS/MS and GC-MS as analytical tools for the determination of steroids in biological fluids. Application of soft ionization techniques in LC-MS/MS has become the method of choice for analyzing intact conjugated steroids. Sample preparation for LC-MS/MS normally includes protein precipitation and solid phase extraction. Electron impact ionization applied in GC-MS fragments the molecules extensively and generates mass spectra those are “fingerprints”. The sample preparation for GC-MS requires a hydrolysis step to remove the conjugate moiety and a derivatization step modifying the physico-chemical properties of steroids to improve the steroid stability and volatility.

Steroid hormones are crucially involved in the regulation of human pregnancy. Amniotic fluid which is swallowed and excreted by the fetus reflects the steroidal milieu of the maternal-feto-placental unit. A new LC-MS/MS assay was developed to determine 6 free and 14 sulfated steroids in amniotic fluid of mid-gestation. The performance of the method was fully evaluated. The method showed good linearity ($r \geq 0.99$) and recovery (100% ± 15%) for all analytes. The limits of quantification ranged from 0.2 to 1.7 ng/mL. Intra-day and inter-day accuracy and precision were below 15% for all analytes. The method requires 600 µL of amniotic fluid. The study provided reference data on multiple unconjugated and sulfated steroids via LC-MS/MS in amniotic fluid. Most of these steroids have been quantified in amniotic fluid for the first time by LC-MS/MS. Highest concentrations in amniotic fluid of mid-gestation were found for progesterone, 16OH-DHEAS and androsterone sulfate. Of all steroid hormones investigated, only testosterone showed a significant sex difference ($p < 0.0001$). The correlations of concentrations of steroid confirmed the classical steroid pathway and a sulfated steroid pathway in the maternal-feto-placental unit.

Steroids with a 3β-hydroxy-5-ene or saturated ring structure are difficult to ionize in LC–MS/MS. A comprehensive GC-MS metabolome approach was then employed to measure more steroids in amniotic fluid of mid-gestation. In total, 52 steroids including pregnenolone and 17-OH-pregnenolone metabolites, DHEA and its metabolites, progesterone and 17-OH-progesterone metabolites, sex hormones as well as corticosterone and cortisol metabolites were profiled by a targeted GC-MS method. The

sum of pregnenolone and 17-OH-pregnenolone metabolites showed the highest concentrations of all groups of steroids analyzed. In particular, 5-Pregnene-3β,20α,21-triol (mean ± SD: 45.3 ± 23.2 ng/mL) and 5-Pregnene-3β,21-diol-20-one (43.9 ± 24.0 ng/mL) showed the highest levels within this group. The sum of cortisone metabolites was clearly higher than the sum of cortisol metabolites. This indicated a preference of cortisol inactivation by the feto-placental unit. Only testosterone showed a significant sex difference ($p < 0.0001$). Our study also showed that the steroid hormones in AF of mid-gestation were not indicative of an active "backdoor" pathway. Our basic data provided reference intervals for further studies characterizing diseases relevant to steroid metabolism.

To the best of our knowledge, only a few groups have investigated physiological concentrations of steroid glucuronides in human urine by MS based techniques (Table 1 in publication 3). Moreover, validation data were sometimes missing. Therefore, we developed and validated a new LC-MS/MS method for determining urinary concentrations of steroid glucuronides. The method can simultaneously quantify androgen glucuronides, estrogen glucuronides and cortisol glucuronide requiring 2 mL of urine. The performance of the assay was studied at three quality control levels for all compounds. Intra-day and inter-day accuracy (recovery, RE: 100% ± 15%) and precision (CV ≤ 15%) met criteria for all analytes. The method was applied to urine samples of different age groups. Free and sulfated steroids were also measured by LC-MS/MS. In addition, total concentrations of steroids were measured by GC-MS after enzymatic hydrolysis. In general, the levels of the free fractions were by far lower than those of the corresponding conjugated fractions. For example, the levels of unconjugated androsterone were 2.4 nmol/L (median) in the age group of 15–20-year-old, while those of sulfated and glucuronidated conjugates were 1171.2 and 4368.9 nmol/L, respectively. Moreover, the sums of the glucuronidated, sulfated and free forms of an analyte showed good correspondence with its total amount determined after enzymatic hydrolysis by GC-MS.

In our studies, LC-MS/MS with soft ionization techniques was used to detected unconjugated steroids and sulfated steroids in amniotic fluid and glucuronidated steroids in urine. GC-MS with hard ionization technique was utilized to characterize and quantify steroids more comprehensively in amniotic fluid. Therefore, LC-MS/MS and GC-MS are not competitive but complementary techniques.

6 Zusammenfassung

In unseren Studien verwendeten wir sowohl LC-MS/MS als auch GC-MS als Analyseinstrumente für die Bestimmung von Steroiden in biologischen Flüssigkeiten. Die Anwendung von Soft-Ionisationstechniken in der LC-MS/MS hat sich zur Methode der Wahl für die Analyse intakter konjugierter Steroide entwickelt. Die Probenvorbereitung für LC-MS/MS umfasst in der Regel eine Proteinausfällung und eine Festphasenextraktion. Die bei der GC-MS angewandte Elektronenstoßionisation fragmentiert die Moleküle weitgehend und erzeugt Massenspektren, die "Fingerabdrücke" sind. Die Probenvorbereitung für GC-MS erfordert einen Hydrolyseschritt, um die konjugierte Einheit zu entfernen, und einen Derivatisierungsschritt, der die physikalisch-chemischen Eigenschaften der Steroide verändert, um die Stabilität und Flüchtigkeit der Steroide zu verbessern.

Steroidhormone sind entscheidend an der Regulation der menschlichen Schwangerschaft beteiligt. Fruchtwasser, das vom Fötus verschluckt und ausgeschieden wird, spiegelt das steroidale Milieu der mütterlich-feto-plazentaren Einheit wider. Ein neuer LC-MS/MS-Assay wurde entwickelt, um 6 freie und 14 sulfatierte Steroide im Fruchtwasser der mittleren Schwangerschaft zu bestimmen. Die Leistungsfähigkeit der Methode wurde vollständig evaluiert. Die Methode zeigte gute Linearität und Wiederfindung für alle Analyten. Die Bestimmungsgrenzen reichten von 0,2 bis 1,7 ng/mL. Intra- und inter-Tagesgenauigkeit und Präzision lagen für alle Analyten unter 15 %. Die Methode erfordert 600 µL Fruchtwasser. Die Studie lieferte Referenzdaten zu mehreren unkonjugierten und sulfatierten Steroiden mittels LC-MS/MS in Fruchtwasser. Die meisten dieser Steroide wurden zum ersten Mal in Fruchtwasser mittels LC-MS/MS quantifiziert. Die höchsten Konzentrationen im Fruchtwasser der mittleren Trächtigkeit wurden für Progesteron, 16OH-DHEAS und Androsteronsulfat gefunden. Von allen untersuchten Steroidhormonen zeigte nur Testosteron einen signifikanten Geschlechtsunterschied ($p < 0.0001$). Die Korrelationen der Steroidkonzentrationen bestätigten den klassischen Steroidpfad und einen sulfatierten Steroidpfad in der maternal-feto-plazentaren Einheit.

Steroide mit einer 3β-Hydroxy-5-en oder gesättigten Ringstruktur sind in der LC-MS/MS schwer zu ionisieren. Ein umfassender GC-MS-Metabolom-Ansatz wurde dann

eingesetzt, um mehr Steroide im Fruchtwasser in der Mitte der Schwangerschaft zu messen. Insgesamt wurden 52 Steroide, darunter Pregnenolon und 17-OH-Pregnenolon-Metaboliten, DHEA und seine Metaboliten, Progesteron und 17-OH-Progesteron-Metaboliten, Sexualhormone sowie Corticosteron- und Cortisol-Metaboliten durch eine gezielte GC-MS-Methode profiliert. Die Summe von Pregnenolon und 17-OH-Pregnenolon-Metaboliten zeigte die höchsten Konzentrationen aller untersuchten Steroidgruppen. Insbesondere 5-Pregnen-3β,20α,21-triol (Mittelwert ± SD: 45,3 ± 23,2 ng/mL) und 5-Pregnen-3β,21-diol-20-one (43,9 ± 24,0 ng/mL) zeigten die höchsten Werte innerhalb dieser Gruppe. Die Summe der Cortison-Metabolite war deutlich höher als die Summe der Cortisol-Metabolite. Dies deutet auf eine Bevorzugung der Cortisol-Inaktivierung durch die feto-plazentare Einheit hin. Nur Testosteron zeigte einen signifikanten Geschlechtsunterschied ($p < 0,0001$). Unsere Studie zeigte auch, dass die Steroidhormone im AF der mittleren Gestation nicht auf einen aktiven "Hintertür"-Weg hinweisen. Unsere Basisdaten lieferten Referenzintervalle für weitere Studien zur Charakterisierung von Krankheiten, die für den Steroidstoffwechsel relevant sind.

Soweit wir wissen, haben nur wenige Gruppen physiologische Konzentrationen von Steroidglukuroniden im menschlichen Urin mit MS-basierten Techniken untersucht. Außerdem fehlten manchmal Validierungsdaten. Daher haben wir eine neue LC-MS/MS-Methode zur Bestimmung der Steroidglucuronid-Konzentrationen im Urin entwickelt und validiert. Mit der Methode können Androgenglucuronide, Östrogenglucuronide und Cortisolglucuronid gleichzeitig quantifiziert werden, wobei 2 ml Urin benötigt werden. Die Leistungsfähigkeit des Assays wurde bei drei Qualitätskontrollstufen für alle Verbindungen untersucht. Die Intra- und Inter-Tagesgenauigkeit (Wiederfindung, RE: 100 % ± 15 %) und die Präzision (CV ≤ 15 %) erfüllten die Kriterien für alle Analyten. Die Methode wurde auf Urinproben verschiedener Altersgruppen angewandt. Freie und sulfatierte Steroide wurden ebenfalls mittels LC-MS/MS gemessen. Darüber hinaus wurden die Gesamtkonzentrationen der Steroide nach enzymatischer Hydrolyse mittels GC-MS gemessen. Im Allgemeinen waren die Konzentrationen der freien Fraktionen bei weitem niedriger als die der entsprechenden konjugierten Fraktionen. So betrug beispielsweise der Gehalt an nicht konjugiertem Androsteron in der Altersgruppe der 15- bis 20-Jährigen 2,4 nmol/L (Median), während der Gehalt an sulfatierten und glucuronidierten Konjugaten 1171,2 bzw. 4368,9 nmol/L betrug. Darüber hinaus zeigten die Summen der glucuronidierten, sulfatierten und freien Formen eines Analyten eine

gute Übereinstimmung mit seiner Gesamtmenge, die nach enzymatischer Hydrolyse mittels GC-MS bestimmt wurde.

In unseren Studien wurde LC-MS/MS mit weichen Ionisierungstechniken verwendet, um unkonjugierte Steroide und sulfatierte Steroide im Fruchtwasser und glucuronidierte Steroide im Urin nachzuweisen. GC-MS mit harter Ionisierungstechnik wurde zur umfassenderen Charakterisierung und Quantifizierung der Steroide im Fruchtwasser eingesetzt. Daher sind LC-MS/MS und GC-MS keine konkurrierenden, sondern ergänzende Techniken.

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9 Erklärung zur Dissertation

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.

Giessen, 09.12.2022 _____

Ort, Datum Unterschrift

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11 Resume

The curriculum vitae was removed from the electronic version of the dissertation.

Publications

- **R. Wang**, M.F. Hartmann, S.A. Wudy. Targeted LC-MS/MS analysis of 15 steroid glucuronides in human urine. *Journal of Steroid Biochemistry and Molecular Biology*. 2021 Jan; 205: 105774. doi:10.1016/j.jsbmb.2020.105774

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