Název projektu:

Steroidní metabolom a vznik těhotenských patologií

INFORMOVANÝ SOUHLAS

Jméno a příjmení:	**r. č.:**

Vážená paní,

dovolujeme si Vás oslovit s prosbou o pomoc při výzkumu který zkoumá tzv. neuroaktivní a další bioaktivní steroidy, látky tělu vlastní, a porovnává jejich hladiny v průběhu těhotenství. Účelem projektu je zjistit, zda existují rozdíly v případě dvojčat a jednočetným těhotenstvím, resp. zda existují rozdíly mezi jednotlivými dvojčaty v jednom těhotenství. Hladiny neuroaktivních steroidů se v případě těhotenských komplikací (např. potrácení, předčasný porod atd.) liší, ale nevíme, zda je rozdíl i v případě vícečetného těhotenství. To je rizikové z pohledu předčasného porodu, ale přesná příčina zatím není známá.

Jestliže se rozhodnete pro účast ve studii, požádáme Vás 1x během těhotenství o odběr krve ze žíly v loketní jamce a současně Vás požádáme o možnost odběru malého množství pupečníkové krve v období těsně po porodu placenty. Tato krev je jinak považována za biologický odpad a náležitě s placentou likvidována. O spolupráci na projektu chceme požádat kromě Vás asi 100 dalších pacientek.
Vzorky krve budou společně s podobnými vzorky od jiných pacientek zpracovány v zabezpečených laboratořích Endokrinologického ústavu Akademie věd ČR. Ve vzorcích krve budou stanoveny hladiny složek tzv. steroidního metabolomu a budou analyzovány jejich funkce. Tato vyšetření nám pomůžou porovnat, zda a jaký souvis je mezi hladinami neuroaktivních steroidů, které by mohly vést ke spuštění těhotenských komplikací. Zbylá krev bude anonymně uložena v tzv. biobance pro účely případného budoucího výzkumu. Odběr krve nijak neovlivní Váš zdravotní stav ani zdravotní stav Vašeho dítěte/dětí.

Vaše účast v tomto projektu je zcela dobrovolná. Účast můžete odmítnout nebo máte možnost z projektu kdykoli odstoupit i bez udání důvodu. Neovlivní to Váš vztah s lékařem, který Vám poskytne nejlepší dostupnou léčbu. Jestliže zrušíte svůj souhlas s účastí ve studii před odesláním vzorků do laboratoře, Váš lékař zařídí jejich znehodnocení.
Dbáme o to, aby byl výzkum provázen vysokým stupněm zachování důvěrnosti. Vaše osobní údaje budou chráněny a používány v souladu s předpisy o ochranně osobních údajů dle platných zákonů ČR. Vaše vzorky budou v laboratoři označeny kódem, nikoliv osobními údaji (například jménem). Z tohoto kódu bude omezený počet osob schopen pomocí speciálního klíče identifikovat Váš věk, datum odběru krve a údaj o Vašem těhotenství. Údaje získané při zkouškách budou přísně důvěrné. V centrálním protokolu budou všechny informace uvedeny anonymně a k vyhodnocení se budou používat pouze souhrnné údaje od všech žen.

Za účast v tomto projektu Vám nepřipadá žádná odměna. Váš biologický materiál bude použit pouze k výzkumným účelům a nebude nikdy prodán.
V případě, že se rozhodnete studie zúčastnit, budete požádána, abyste podepsala tento Informovaný souhlas. Účast ve studii můžete odmítnout nebo máte možnost z projektu kdykoli odstoupit i bez udání důvodu.
S dotazy ohledně projektu se obracejte na:
Lékaři: MUDr. A. Černý, prof. MUDr. Antonín Pařízek, CSc.
Tel. č. 224 967 012
Adresa: Gynekologicko-porodnická klinika VFN a 1.LF UK, Apolinářská 18, 128 51 Praha 2

Přečetla jsem si všechny uvedené informace a porozuměla jsem jim. Měla jsem možnost diskutovat o nich a ptát se na ně. Všechny mé dotazy mi byly uspokojivě zodpovězeny. Dobrovolně souhlasím se svou účastí v projektu. Jsem si vědoma toho, že moje účast je naprosto dobrovolná.
Souhlasím se sběrem, použitím a zveřejněním lékařských informací o mé osobě v souladu s účely zde popsanými.

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Steroid Metabolome Analysis in Dichorionic Diamniotic Twin Pregnancy

Andrej Černý [1,*], Martin Hill [2], Michala Vosátková [2], Zdeněk Laštůvka [1] and Antonín Pařízek [1]

[1] Department of Gynaecology, Obstetrics and Neonatology, First Faculty of Medicine, Charles University and General University Hospital in Prague, 12808 Prague, Czech Republic; lastuvkaz@gmail.com (Z.L.); aparizek@seznam.cz (A.P.)
[2] Department of Steroids and Proteofactors, Institute of Endocrinology, 11000 Prague, Czech Republic; mhill@endo.cz (M.H.); mvosatkova@endo.cz (M.V.)
* Correspondence: andrej.cerny@gmail.com; Tel.: +420-732-111-459

Abstract: Steroid hormones have diverse roles in pregnancy; some help stabilise pregnancy and influence the stability of pregnancy and the onset of labour. Changes and disorders in steroidogenesis may be involved in several pregnancy pathologies. To date, only a few studies have performed a very limited steroid analysis in multiple pregnancies. Our teams investigated multiple pregnancies regarding the biosynthesis, transport, and effects of steroids. We recruited two groups of patients: pregnant women with multiple pregnancies as the study group, and a control singleton pregnancies group. Blood samples were drawn from the participants and analysed. Information about the mother, foetus, delivery, and newborn was extracted from medical records. The data were then analysed. The gestational age of twin pregnancies during delivery ranged from 35 + 3 to 39 + 3 weeks, while it was 38 + 1 to 41 + 1 weeks for the controls. Our findings provide answers to questions regarding the steroidome in multiple pregnancies. Results demonstrate differences in the steroidome between singleton and twin pregnancies. These were based on the presence of two placentae and two foetal adrenal glands, both with separate enzymatic activity. Since every newborn was delivered by caesarean section, analysis was not negatively influenced by changes in the steroid metabolome associated with the spontaneous onset of labour.

Keywords: foetomaternal steroidome; neuroactive steroids; multiple pregnancy; pregnancy complications

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1. Introduction

Steroid hormones are synthesised in adrenal, gonadal, cerebral, and liver tissues. The placenta and the foetal adrenal zone (fetoplacental unit) are primarily responsible for the production of steroid hormones during pregnancy. A set of all steroid metabolites is called the "steroid metabolome". Based on the mechanism of action, there are two kinds of steroids:

1. Genomic: these bind to intracellular receptors or directly to DNA. This effect is slow; the response usually occurs hours or days later. They act as transcription factors that activate or suppress gene expression.
2. Non-genomic: Their effect is associated with neurotransmitter receptors in the cytoplasmic membrane. These receptors affect ion channels and neuronal excitability. Changes based on this principle are considerably faster, ranging from seconds to milliseconds. Steroids with this effect are called "neuroactive steroids" or "neurosteroids", and their mechanism of action was described between 1980 and 1990 [1,2].

Neurosteroids are steroid hormones synthesised in the central and peripheral nervous system. Neuroactive steroids are steroids that have neuroactive effects but can be synthesised in areas other than nervous tissue. Pregnane-type steroids, such as allopregnanolone,

isopregnanolone, pregnanolone, and epipregnanolone, play an important role in pregnancy. Neuroactive steroids bind to several receptor types. GABAA-r (type A γ-aminobutyric acid receptor) and NDMA-r (N-methyl-D-aspartate receptor) are mostly affected. GABAA receptors influence chloride ion channels, and NDMA receptors influence calcium ion channels. These receptors are also located in the uterine muscle. This partially explains the effects of uterine contractility [3].

The endocrine activity of the foetoplacental unit during pregnancy is high. The adrenal gland and the foetal zone are the main organs for neuroactive steroid synthesis in the foetal body. Foetal zone activity is stimulated by the placental corticotropin-releasing hormone (CRH). The transport of hormones and their precursors within the foetoplacental unit is necessary due to the lack of enzymes in the placenta or foetus. At the end of the process, sufficient hormones for foetal development are produced [4].

1.1. Clinical Impact of Steroid Metabolome Studies

Several enzymes are involved in the synthesis and metabolism of steroid hormones. Defects in these systems can lead to complications during pregnancy. Neuroactive steroids are important for maintaining the stability of the foetoplacental unit. Changes in these levels are closely linked with the timing of delivery [5]. Previous research led by our team also compared the steroid metabolome between women who delivered spontaneously and those who delivered by caesarean section [6].

CRH is an important proteohormone involved in the timing of labour onset. Most of the CRH is produced in the placenta during pregnancy. Its influence on the foetoplacental unit appears to play a key role in the onset of labour. Some authors consider CRH to work as a "placental clock". CRH levels are the highest at the beginning of delivery—almost ten thousand times higher than during pregnancy [7–9]. Right before the delivery, "relaxing" isoforms of receptors for CRH are converted to those that influence myometrium contractility. CRH also influences the effect of oxytocin and prostaglandin F2α. These processes explain the timing of delivery [10–12].

Hill's study attempted to create a model for predicting labour onset. This study selected a group of neuroactive steroids that could be used as predictors of labour onset. Although the prediction models were better for umbilical blood, the model based on maternal blood was still very effective. In contrast to umbilical blood, using maternal blood for prediction is practically feasible and ethically acceptable [8].

The relationship between intrahepatic cholestasis during pregnancy (ICP) and steroid metabolome changes is well-known. In ICP cases, a lower production of adrenocorticotropic hormone (ACTH) and cortisone has been observed [13]. This may lead us to conclude that the CRH–ACTH axis may be altered. At the same time, the CRH–ACTH axis functioning is associated with the production of neuroactive steroids [14]. ICP in pregnancy has been studied by our team in several studies [15,16].

Pregnane steroids are involved in the regulation of blood pressure. Some authors reported significantly higher levels of allopregnanolone in women with chronic hypertension. In cases in which hypertension was complicated by preeclampsia, allopregnanolone levels were even higher. This study was based on a study from 1979 that investigated the relationship between levels of progesterone and 5α-dihydroprogesterone in patients with gestational hypertension. However, this relationship was not confirmed because the study was based only on the analysis of maternal serum and not umbilical cord blood. Progesterone levels in the amniotic fluid are significantly higher in women with later onset of preeclampsia [17,18]. This complication is caused by the dysregulation of angiogenic and anti-angiogenic growth factors in the placenta. Defects in placental oestrogen synthesis are believed to be one of the factors leading to preeclampsia [19].

Neuroactive steroids affect foetal growth, and alterations in the steroid metabolome may induce foetal growth restriction [20]. The effects of neuroactive steroids on the foetal and maternal central nervous systems are significant. The foetus' central nervous system is in a state of permanent sleep, and foetal awareness is suppressed. Neuroactive steroids

play an important role as neuroprotective agents. Allopregnanolone levels rise during acute hypoxia and protect the foetal brain during delivery. However, these mechanisms do not function during chronic hypoxia, and the foetus is thus at a higher threat [21–23].

Further, lower levels of neuroactive steroids are associated with a higher risk of depression during pregnancy or the postpartum period [24,25]. Coincidence between depression and allopregnanolone levels can be observed in the third trimester and later [26]. Pregnane steroids may alleviate pain perception during labour via negative modulation of T-type calcium channels [27].

Neuroactive steroids may also be used as therapeutic agents. Several projects have studied the benefits of neuroactive steroids for treating and preventing psychiatric diseases, such as depression, migraine, anxiety, panic attacks, and insomnia. The US Food and Drug Administration approved brexanolone (allopregnanolone) as an efficient anti-depression and anti-anxiety drug [28].

Neuroactive steroid therapy has been successfully used to treat nicotine and cocaine abuse [29]. Moreover, brexanolone has been investigated as part of the treatment for post-COVID acute respiratory distress syndrome [30]. In addition, ganaxolone (an allopregnanolone derivative) can potentially influence the neurological deficiency caused by premature labour [31].

1.2. Multiple Pregnancies

Multiple pregnancies are a reproductive health abnormality. This abnormality can however lead to serious obstetric complications. The population frequency was estimated using Hellin's law. This rule approximates the frequency of twin pregnancy to 1:85, triplets to $1:85^2$, and quadruplets to $1:85^3$. Countries with higher frequency of assisted reproduction methods show higher frequencies of multiple pregnancies in their population.

Patients with multiple pregnancies have a significantly higher risk for serious pregnancy-related complications. Premature labour is one of the most threatening high-risk pathologies in multiple pregnancies [32,33]. Besides that, multiple pregnancy foetuses are in greater danger of intrauterine growth restriction in at least one of the foetuses, twin-to-twin transfusion syndrome, respiratory distress syndrome (RDS), and intrauterine stillbirth or abortion [34–36].

The endocrinology of multiple pregnancies has been the focus of several studies. The basis of these studies was the "twin testosterone transfer" hypothesis. This hypothesis states that androgen overproduction by the male foetus negatively affects the female foetus and induces masculine behavioural patterns, appearance, and cognition. This effect was also observed in mice. A Dutch experiment showed that maternal testosterone had no influence on female foetuses during pregnancy. Some authors believe that androgens from mothers are fully converted into oestrogens by the placenta [37]. However, other studies do not support or disconfirm this hypothesis [38,39]. In addition, foetuses in multiple pregnancies are not influenced by male siblings differently than when both foetuses are female [40,41].

In collaboration with the Department of Steroids and Proteofactors of the Institute of Endocrinology in Prague, our research also focused on investigating the biosynthesis, transport, and effects of steroids in multiple pregnancies. To date, no study has comprehensively analysed the steroidome in children and mothers with multiple pregnancies [42]. Therefore, our research aimed to clarify the relationships between foetuses and mothers, and between foetuses from the point of view of steroid synthesis and transport, as well as the physiology and pathophysiology of human pregnancy and childbirth. The study focused on differences based on arteriovenous differentiation.

1.3. Aims

The aims of the study of the steroid metabolome in dichorionic diamniotic twins were:

to describe details of the components of the steroid metabolome in twins,
to show the difference between multiple and singleton pregnancies,

to show the differences between male and female foetuses because the metabolism of the precursors is different.

Based on these aims and knowledge, we aimed to establish a model of steroidogenesis in multiple pregnancies in the same manner as that in singleton pregnancies.

2. Results

The list of metabolites analysed and their values for cord arterial blood and cord venous blood in singleton and twin pregnancies are given in Table 1 and for maternal venous blood in Table 2. Supplementary Tables S1–S3 show the results of the OPLS models for distinguishing twin from singleton pregnancies based on steroid levels in umbilical arterial blood (Table S1), umbilical venous blood (Table S2), and maternal venous blood (Table S3). Supplementary Tables S4–S6 display statistically significant differences between female and male foetuses in terms of steroid levels in umbilical arterial blood (Table S4), umbilical venous blood (Table S5), and maternal venous blood (Table S6). Values for the control group are included. After excluding samples that did not meet the criteria, we used 81 venous and arterial foetal blood samples, combined with 42 maternal blood samples. The control group included 47 foetal and maternal blood samples. The gestational age of twin pregnancies during delivery varied from 35 + 3 to 39 + 3 weeks. Controls were delivered at gestational ages of 38 + 1 and 41 + 1 weeks.

Table 1. Steroid differences between dichorionic diamniotic twin pregnancy and singleton pregnancy in umbilical venous blood.

Steroid	Umbilical Artery Pregnancy Type				Umbilical Vein Pregnancy Type			
	Singleton	Twin	*p*	η_p^2	Singleton	Twin	*p*	η_p^2
Pregnenolone [nM]	37.1 (31.7, 43.7)	49.2 (43.1, 56.8)	0.084	0.076	**34.1 (29.4, 39.5)**	**47.3 (43.2, 51.9)**	**0.015**	**0.119**
Pregnenolone sulphate [μM]	**4.19 (3.46, 5.02)**	**2.52 (2.13, 2.94)**	**0.01**	**0.165**	**3.7 (3.03, 4.49)**	**2.14 (1.86, 2.44)**	**0.004**	**0.157**
17-Hydroxypregnenolone [nM]	23.9 (17.6, 32.3)	39.3 (31.1, 50.1)	0.092	0.073	8.21 (5.66, 11.8)	10.8 (8.66, 13.4)	0.397	0.015
17-Hydroxypregnenolone sulphate [μM]	1.98 (1.4, 2.72)	1.8 (1.4, 2.28)	0.766	0.002	1.82 (1.35, 2.45)	1.81 (1.5, 2.18)	0.979	<0.001
16α-Hydroxypregnenolone [nM]	**15.3 (12.6, 18.7)**	**30.3 (25.3, 36.8)**	**0.002**	**0.229**	**6.92 (5.93, 8.15)**	**10.2 (9.15, 11.5)**	**0.013**	**0.125**
20α-Dihydropregnenolone [nM]	3.92 (3.35, 4.68)	5.53 (4.78, 6.54)	0.055	0.096	2.89 (2.36, 3.49)	3.97 (3.56, 4.42)	0.058	0.075
20α-Dihydropregnenolone sulphate [μM]	2.22 (1.83, 2.66)	1.8 (1.56, 2.07)	0.25	0.038	2.47 (1.98, 3.05)	1.89 (1.64, 2.17)	0.181	0.037
Dehydroepiandrosterone [nM]	8.05 (6.03, 10.9)	9.38 (7.51, 11.8)	0.589	0.008	2.56 (2.14, 3.12)	2.38 (2.14, 2.66)	0.646	0.005
DHEA sulphate [μM]	**3.28 (2.78, 3.87)**	**2.16 (1.91, 2.45)**	**0.011**	**0.166**	**3.28 (2.74, 3.87)**	**1.79 (1.55, 2.05)**	**0.001**	**0.199**
7α-Hydroxy-DHEA [nM]	0.798 (0.652, 0.968)	0.907 (0.78, 1.05)	0.494	0.013	0.458 (0.36, 0.593)	0.437 (0.378, 0.508)	0.828	0.001
7-oxo-DHEA [nM]	1.23 (0.943, 1.57)	1.78 (1.51, 2.09)	0.102	0.071	1.03 (0.783, 1.34)	1.16 (0.99, 1.37)	0.598	0.006
7β-Hydroxy-DHEA [nM]	0.259 (0.206, 0.322)	0.313 (0.265, 0.368)	0.36	0.023	0.174 (0.126, 0.247)	0.125 (0.103, 0.151)	0.241	0.029
Androstenediol [nM]	0.428 (0.328, 0.559)	0.438 (0.359, 0.536)	0.924	<0.001	**0.126 (0.101, 0.152)**	**0.178 (0.162, 0.194)**	**0.029**	**0.101**
Androstenediol sulphate [μM]	5.01 (3.84, 6.4)	4.17 (3.41, 5.04)	0.456	0.015	5.62 (4.29, 7.17)	4.67 (3.91, 5.51)	0.438	0.013
5-Androstene-3β,7α,17β-triol [pM]	42.1 (24.8, 64.5)	71.8 (54.1, 92.4)	0.176	0.049	6.59 (3.71, 11.2)	11 (8.08, 15)	0.268	0.026
5-Androstene-3β,7β,17β-triol [pM]	8.76 (4.36, 16.6)	24.8 (15.9, 38.6)	0.084	0.079	2.12 (1.23, 3.63)	3.57 (2.58, 5.03)	0.273	0.026
5-Androstene-3β,16α,17β-triol [nM]	2.58 (1.83, 3.6)	3.1 (2.41, 3.98)	0.559	0.009	2.74 (2.12, 3.59)	3.04 (2.59, 3.58)	0.659	0.004
5-Androstene-3β,16α,17β-triol sulphate [nM]	518 (435, 621)	557 (493, 631)	0.665	0.005	547 (453, 659)	509 (455, 569)	0.669	0.004
Progesterone [μM]	0.991 (0.719, 1.36)	1.44 (1.14, 1.82)	0.216	0.04	**1.76 (1.4, 2.22)**	**2.74 (2.38, 3.15)**	**0.035**	**0.094**
17-Hydroxyprogesterone [nM]	50.1 (39.3, 63.4)	66.3 (55.7, 78.9)	0.213	0.041	73.8 (58.9, 89.6)	88.8 (79, 99)	0.286	0.025
17,20α-Dihydroxy-4-pregnene-3-one [nM]	8.82 (7.13, 10.9)	13 (11.1, 15.3)	0.061	0.091	11.8 (9.25, 15.1)	15.1 (13, 17.6)	0.267	0.026
17,20α-Dihydroxy-4-pregnene-3-one, conjugated [nM]	17.4 (14.3, 21.5)	20.9 (17.8, 24.7)	0.359	0.022	20.1 (16.5, 24.6)	22.3 (19.7, 25.3)	0.552	0.007
16α-Hydroxyprogesterone [nM]	63 (47.7, 83.5)	97.5 (78.5, 122)	0.112	0.065	96.3 (75.5, 123)	135 (116, 158)	0.129	0.048
20α-Dihydroprogesterone [nM]	**67.9 (54.9, 83.5)**	**110 (94.1, 128)**	**0.018**	**0.139**	**46.4 (37.6, 56.6)**	**69.1 (61.8, 77)**	**0.022**	**0.106**
20α-Dihydroprogesterone, conjugated [nM]	62.4 (50.5, 77.9)	75.1 (63.9, 88.9)	0.374	0.021	69.8 (56.5, 85)	74.3 (65.8, 83.6)	0.722	0.003
Androstenedione [nM]	2.42 (2.01, 2.92)	2.79 (2.43, 3.22)	0.423	0.018	2.45 (1.94, 3.17)	2.32 (2.02, 2.69)	0.796	0.001
Testosterone [pM]	223 (99.6, 491)	297 (168, 524)	0.699	0.004	52.4 (29.5, 94.6)	60.1 (42.6, 85.7)	0.787	0.002
Testosterone, conjugated [nM]	34.2 (27.7, 42.9)	34.8 (30, 40.7)	0.933	<0.001	41 (31.9, 51.6)	39.1 (33.5, 45.3)	0.829	<0.001
16α-Hydroxytestosterone [nM]	8.96 (6.93, 11.5)	10.9 (9.08, 13)	0.407	0.019	8.31 (6.24, 10.9)	9.79 (8.3, 11.5)	0.497	0.01
16α-Hydroxytestosterone, conjugated [nM]	11.1 (8.54, 14.3)	11.5 (9.45, 13.8)	0.9	<0.001	10.1 (7.65, 13.2)	11.3 (9.53, 13.3)	0.656	0.004
Epitestosterone, conjugated [nM]	360 (299, 435)	362 (317, 416)	0.969	<0.001	429 (357, 515)	372 (331, 417)	0.384	0.016
5α-Dihydrotestosterone [pM]	58.2 (32.2, 103)	61.6 (40.4, 92.9)	0.916	<0.001	32.5 (17.9, 56.2)	42.1 (29.8, 58.8)	0.602	0.006
Estrone [nM]	16.1 (10, 25.2)	16 (11.4, 22.2)	0.994	<0.001	32 (22, 43.8)	49 (41.1, 57.5)	0.121	0.049
Estrone sulphate [nM]	90.9 (66.3, 127)	79.8 (63.2, 102)	0.667	0.005	86.4 (60.5, 124)	83.5 (66.9, 104)	0.913	<0.001

Table 1. *Cont.*

Steroid	Umbilical Artery Pregnancy Type				Umbilical Vein Pregnancy Type			
	Singleton	Twin	p	η_p^2	Singleton	Twin	p	η_p^2
Estradiol [nM]	12.7 (11.4, 14.3)	14.2 (12.8, 15.9)	0.356	0.028	**18.6 (17.2, 20.1)**	**23.8 (22.5, 25.2)**	**0.002**	**0.228**
Estradiol sulphate [nM]	9.38 (7.34, 11.9)	12.5 (10.6, 14.7)	0.192	0.047	11.7 (9.41, 14.4)	10.3 (8.99, 11.8)	0.52	0.009
Estriol [nM]	161 (96.9, 266)	230 (158, 334)	0.455	0.015	374 (283, 503)	383 (323, 457)	0.927	<0.001
Estriol sulphate [μM]	3.57 (3.01, 4.27)	3.49 (3.08, 3.97)	0.888	<0.001	3.64 (2.97, 4.37)	2.89 (2.51, 3.3)	0.216	0.032
5α-Dihydroprogesterone [nM]	**124 (109, 142)**	**229 (198, 269)**	**<0.001**	**0.309**	**115 (98.5, 135)**	**289 (254, 334)**	**<0.001**	**0.415**
Allopregnanolone [nM]	**17 (14.5, 19.9)**	**31.1 (27.3, 35.5)**	**<0.001**	**0.29**	**14.4 (11.8, 17.4)**	**30.7 (27.5, 34.4)**	**<0.001**	**0.315**
Allopregnanolone sulphate [nM]	346 (264, 453)	503 (412, 616)	0.146	0.056	411 (314, 542)	552 (464, 659)	0.237	0.029
Isopregnanolone [nM]	**23.9 (20.3, 28.1)**	**56.2 (49.3, 64.2)**	**<0.001**	**0.444**	**17.3 (14, 21.2)**	**38.3 (34.7, 42.2)**	**<0.001**	**0.356**
Isopregnanolone sulphate [nM]	374 (300, 471)	426 (363, 503)	0.533	0.011	385 (310, 483)	385 (338, 441)	1	<0.001
5β-Dihydroprogesterone [nM]	27.5 (23.6, 32.8)	29.1 (25.9, 33.2)	0.719	0.004	**26.1 (21.9, 30.9)**	**35.4 (32.2, 38.9)**	**0.04**	**0.087**
Pregnanolone [nM]	24.5 (18.4, 32.3)	26.2 (21.2, 32.1)	0.806	0.002	**9.89 (7.87, 12.2)**	**15.1 (13.5, 16.9)**	**0.022**	**0.109**
Pregnanolone, conjugated [nM]	289 (226, 366)	330 (277, 390)	0.556	0.009	344 (267, 433)	374 (322, 430)	0.689	0.003
Epipregnanolone [nM]	1.85 (1.49, 2.3)	2.04 (1.73, 2.39)	0.643	0.006	1.18 (0.966, 1.43)	1.49 (1.33, 1.66)	0.171	0.04
Epipregnanolone, conjugated [nM]	61.7 (50.9, 75.5)	78 (67.2, 91.4)	0.221	0.04	66.6 (55.6, 80.2)	74.1 (66.1, 83.3)	0.518	0.009
5α,20α-Tetrahydroprogesterone [nM]	**49 (41.2, 58.5)**	**103 (88.2, 122)**	**<0.001**	**0.313**	**39.7 (31.1, 48.6)**	**79.1 (72.7, 85.7)**	**<0.001**	**0.326**
5α,20α-Tetrahydroprogesterone, conjugated [nM]	221 (153, 322)	238 (182, 312)	0.837	0.001	277 (190, 404)	249 (198, 314)	0.754	0.002
5α-Pregnane-3α,20α-diol [nM]	**6.31 (4.92, 8.04)**	**15.3 (12.8, 18.5)**	**<0.001**	**0.286**	**4.02 (2.96, 5.28)**	**10.7 (9.48, 12.1)**	**<0.001**	**0.333**
5α-Pregnane-3α,20α-diol, conjugated [μM]	3.85 (2.77, 5.32)	4.82 (3.81, 6.1)	0.461	0.015	5.02 (3.56, 7.03)	5.55 (4.49, 6.83)	0.74	0.002
5α-Pregnane-3β,20α-diol [nM]	**4.2 (3.59, 4.91)**	**9.23 (8.09, 10.6)**	**<0.001**	**0.407**	**3.75 (3.11, 4.52)**	**7.72 (6.84, 8.74)**	**<0.001**	**0.282**
5α-Pregnane-3β,20α-diol, conjugated [μM]	**2.67 (1.97, 3.56)**	**4.63 (3.81, 5.6)**	**0.04**	**0.109**	3.11 (2.34, 4.07)	4.51 (3.84, 5.27)	0.122	0.049
5β,20α-Tetrahydroprogesterone [nM]	21.9 (18, 26.9)	26.5 (22.7, 31.2)	0.332	0.025	19.4 (15.9, 23.6)	27.1 (24.2, 30.3)	0.054	0.077
5β,20α-Tetrahydroprogesterone, conjugated [nM]	130 (91.7, 182)	88.7 (67.9, 115)	0.246	0.036	174 (127, 235)	98.6 (79, 122)	0.055	0.074
5β-Pregnane-3α,20α-diol [nM]	24.7 (16.5, 35.7)	30.5 (23, 40)	0.55	0.009	6.6 (5.07, 8.63)	8.16 (6.95, 9.62)	0.368	0.017
5β-Pregnane-3α,20α-diol, conjugated [μM]	3.02 (2.27, 3.95)	2.72 (2.2, 3.32)	0.685	0.005	3.3 (2.54, 4.23)	2.8 (2.38, 3.27)	0.474	0.011
5β-Pregnane-3β,20α-diol [nM]	0.589 (0.446, 0.78)	0.818 (0.662, 1.02)	0.222	0.039	0.492 (0.396, 0.616)	0.609 (0.532, 0.7)	0.28	0.025
5β-Pregnane-3β,20α-diol, conjugated [nM]	573 (453, 731)	462 (404, 531)	0.302	0.023	438 (347, 546)	448 (381, 522)	0.916	<0.001
17-Hydroxyallopregnanolone [nM]	**0.55 (0.414, 0.711)**	**1.11 (0.958, 1.29)**	**0.003**	**0.221**	**0.35 (0.275, 0.441)**	**0.767 (0.67, 0.878)**	**<0.001**	**0.247**
17-Hydroxyallopregnanolone sulphate [nM]	**7.57 (6.28, 9.25)**	**11.5 (9.71, 14)**	**0.042**	**0.107**	**7.04 (5.73, 8.7)**	**13.1 (11.3, 15.3)**	**0.003**	**0.175**
17-Hydroxypregnanolone [nM]	1.07 (0.865, 1.32)	1.34 (1.15, 1.57)	0.258	0.035	**0.657 (0.57, 0.756)**	**0.895 (0.823, 0.973)**	**0.016**	**0.126**
17-Hydroxypregnanolone, conjugate [nM]	47.3 (38, 58.4)	62.2 (53.1, 72.7)	0.176	0.048	49.6 (39.5, 62)	64.1 (56, 73.4)	0.2	0.034
5α-Pregnane-3α,17,20α-triol [pM]	**46.5 (38.6, 56.1)**	**93 (79.8, 109)**	**<0.001**	**0.276**	**42.2 (33.1, 53.6)**	**92.1 (78.6, 108)**	**<0.001**	**0.214**
5α-Pregnane-3α,17,20α-triol, conjugated [nM]	21.7 (16.5, 28.9)	29.1 (23.4, 36.7)	0.285	0.03	26.5 (19.8, 35.9)	29.7 (24.7, 35.8)	0.675	0.004
5β-Pregnane-3α,17,20α-triol [nM]	**1.59 (1.39, 1.84)**	**2.14 (1.93, 2.38)**	**0.032**	**0.119**	1.68 (1.42, 1.98)	2 (1.81, 2.2)	0.232	0.031
5β-Pregnane-3α,17,20α-triol, conjugated [nM]	387 (305, 502)	454 (373, 560)	0.518	0.011	417 (299, 595)	465 (377, 581)	0.724	0.003
5α-Androstane-3,17-dione [nM]	0.399 (0.318, 0.51)	0.364 (0.308, 0.434)	0.674	0.005	0.186 (0.151, 0.233)	0.186 (0.164, 0.213)	0.998	<0.001

Table 1. *Cont.*

Steroid	Umbilical Artery Pregnancy Type				Umbilical Vein Pregnancy Type			
	Singleton	**Twin**	***p***	**η_p^2**	**Singleton**	**Twin**	***p***	**η_p^2**
Androsterone [nM]	0.228 (0.181, 0.293)	0.15 (0.129, 0.175)	0.055	0.096	138 (110, 178)	94.3 (83.8, 106)	0.055	0.078
Androsterone sulphate [nM]	65.3 (45.5, 93.9)	58.8 (45.2, 76.4)	0.757	0.003	78.6 (55, 112)	61.2 (49, 76.3)	0.433	0.013
Epiandrosterone [nM]	0.2 (0.142, 0.284)	0.251 (0.196, 0.324)	0.49	0.013	50.3 (36.2, 69)	65.5 (54.7, 78.3)	0.341	0.02
Epiandrosterone sulphate [nM]	41.6 (35.9, 47.9)	31.9 (28.3, 35.8)	0.066	0.089	**40.2 (34.3, 46.5)**	**28.8 (25.6, 32.1)**	**0.029**	**0.096**
Etiocholanolone [pM]	41.3 (33.7, 49.9)	42.2 (36.6, 48.4)	0.901	<0.001	49.6 (41, 60.4)	40.2 (35.8, 45.1)	0.211	0.034
Etiocholanolone sulphate [nM]	9.99 (7.83, 12.8)	7.26 (6.14, 8.6)	0.156	0.055	11.4 (8.49, 15.1)	8.2 (6.73, 9.88)	0.216	0.032
Epietiocholanolone sulphate [nM]	1.96 (0.877, 4.07)	4.73 (2.81, 7.87)	0.199	0.044	3.53 (1.66, 6.77)	5.44 (3.61, 7.99)	0.466	0.011
5α-Androstane-3α,17β-diol [pM]	**11.7 (8.44, 16)**	**27 (22, 33.1)**	**0.005**	**0.198**	12.2 (9.01, 17.3)	8.65 (7.39, 10.2)	0.197	0.037
5α-Androstane-3α,17β-diol,conjugated [nM]	**13.7 (11.3, 16.6)**	**21.3 (18.3, 25.1)**	**0.023**	**0.133**	**15.4 (13.1, 18.3)**	**22.3 (19.9, 25.2)**	**0.023**	**0.103**
5α-Androstane-3β,17β-diol,conjugated [nM]	**4.52 (3.71, 5.52)**	**7.83 (6.7, 9.23)**	**0.007**	**0.182**	**4.52 (3.65, 5.57)**	**9.16 (8.08, 10.4)**	**<0.001**	**0.242**
5β-Androstane-3α,17β-diol,conjugated [nM]	4.38 (3.71, 5.3)	3.75 (3.37, 4.2)	0.318	0.028	4.59 (3.76, 5.51)	4.72 (4.19, 5.29)	0.869	<0.001
5β-Androstane-3β,17β-diol,conjugated [nM]	**0.259 (0.212, 0.319)**	**0.439 (0.376, 0.518)**	**0.011**	**0.172**	0.349 (0.264, 0.437)	0.463 (0.406, 0.521)	0.155	0.043
Cortisol [nM]	137 (115, 163)	130 (117, 145)	0.744	0.002	125 (109, 144)	104 (95, 113)	0.133	0.047
Cortisol [nM]	81.2 (67.8, 96.5)	81.4 (71.9, 91.8)	0.99	<0.001	71.5 (59.8, 85.2)	52.7 (47.1, 58.8)	0.06	0.073
Cortisone [nM]	178 (154, 206)	156 (141, 173)	0.345	0.024	196 (170, 228)	163 (149, 178)	0.147	0.043
Corticosterone [nM]	**5.84 (4.33, 7.9)**	**12 (9.55, 15.4)**	**0.017**	**0.144**	4.07 (2.95, 5.37)	5.3 (4.51, 6.17)	0.283	0.025
11-Deoxycortisol [nM]	16.9 (11.7, 24)	17 (13.1, 21.9)	0.979	<0.001	24.5 (17, 35.4)	18.2 (14.5, 22.8)	0.36	0.018
21-Deoxycortisol [pM]	**138 (92.1, 197)**	**338 (271, 419)**	**0.006**	**0.185**	206 (138, 301)	172 (134, 219)	0.607	0.006
11-Deoxycorticosterone [nM]	3.57 (2.33, 5.3)	7.08 (5.43, 9.14)	0.063	0.091	4.49 (3.45, 5.92)	7.32 (6.17, 8.74)	0.051	0.082
11-Deoxycorticosterone sulphate [nM]	100 (72.6, 141)	81.5 (63.9, 105)	0.505	0.012	117 (80.1, 169)	99.4 (78.5, 125)	0.627	0.005
11β-Hydroxyandrostenedione [nM]	6.6 (5.2, 8.38)	8.99 (7.57, 10.7)	0.172	0.05	5.31 (4.15, 6.81)	5.62 (4.85, 6.53)	0.791	0.002
11β-Hydroxytestosterone [nM]	10.1 (7.94, 12.9)	12.8 (10.8, 15.3)	0.295	0.03	9.72 (7.41, 12.8)	11.9 (10.1, 14.2)	0.398	0.015
11β-Hydroxytestosterone, conjugated, A [nM]	**60.6 (43.9, 82.2)**	**25.5 (19.7, 32.4)**	**0.007**	**0.242**	55.2 (37.9, 78.7)	32 (25.6, 39.5)	0.093	0.076
11β-Hydroxyandrosterone [pM]	55.3 (38.2, 79.1)	96.7 (74.9, 125)	0.101	0.071	36 (25.5, 49.8)	53 (43.7, 64)	0.178	0.038
11β-Hydroxyandrosterone sulphate [nM]	7.24 (5.86, 9.15)	7.01 (5.99, 8.32)	0.881	<0.001	7.78 (6.4, 9.6)	8 (7.05, 9.13)	0.878	<0.001
11β-Hydroxyepiandrosterone [pM]	25.6 (16, 40.4)	51.7 (37.2, 72.1)	0.107	0.069	**17.5 (9.92, 29.8)**	**49.7 (36.2, 68.3)**	**0.03**	**0.095**
11β-Hydroxyepiandrosterone sulphate [nM]	5.64 (3.52, 8.29)	11.2 (8.83, 13.8)	0.05	0.109	5.38 (3.41, 7.93)	9.39 (7.65, 11.4)	0.097	0.061
11β-Hydroxyetiocholanolone [pM]	82.5 (56.4, 119)	53.7 (40.1, 71)	0.236	0.038	77.7 (50.6, 116)	49.8 (37.8, 64.7)	0.241	0.029
11β-Hydroxyetiocholanolone sulphate [nM]	2.83 (2.22, 3.65)	2.47 (2.06, 2.97)	0.559	0.009	2.93 (2.27, 3.8)	2.88 (2.45, 3.39)	0.943	<0.001

The differences between twin and singleton pregnancies for each steroid were evaluated using a linear model consisting of factors Pregnancy type (Twin vs. Singleton) and Gender (Male vs. Female) adjusted for maternal age and gestational age at labour. Significant differences ($p < 0.05$) are in bold, *p*. . .*p*-value, η_p^2. . .effect size (0.01~small, 0.06~medium, >0.14~large).

Table 2. Steroid differences between dichorionic diamniotic twin pregnancy and singleton pregnancy in maternal venous blood.

Steroid	Pregnancy Type		p	η_p^2
	Singleton	Twin		
Pregnenolone [nM]	**6.21 (5.18, 7.42)**	**20.7 (17.1, 25.2)**	**<0.001**	**0.583**
Pregnenolone sulfate [nM]	**229 (189, 284)**	**150 (128, 178)**	**0.036**	**0.143**
17-Hydroxypregnenolone [nM]	2.13 (1.57, 2.8)	3.58 (2.8, 4.49)	0.066	0.116
17-Hydroxypregnenolone sulfate [nM]	4.73 (3.32, 6.76)	8.06 (5.7, 11.6)	0.162	0.066
16α-Hydroxypregnenolone [nM]	**1.24 (1.04, 1.48)**	**4.95 (3.99, 6.25)**	**<0.001**	**0.631**
20α-Dihydropregnenolone [nM]	**2.77 (2.37, 3.25)**	**5.88 (4.87, 7.25)**	**<0.001**	**0.37**
20α-Dihydropregnenolone sulfate [μM]	**0.845 (0.653, 1.1)**	**0.407 (0.317, 0.52)**	**0.01**	**0.208**
Dehydroepiandrosterone [nM]	4.74 (3.35, 6.75)	7.99 (5.67, 11.5)	0.167	0.065
DHEA sulfate [μM]	**0.895 (0.626, 1.33)**	**0.351 (0.26, 0.477)**	**0.012**	**0.199**
7α-Hydroxy-DHEA [nM]	0.343 (0.242, 0.472)	0.487 (0.359, 0.646)	0.297	0.037
7-oxo-DHEA [nM]	**0.636 (0.458, 0.862)**	**1.92 (1.49, 2.44)**	**<0.001**	**0.334**
7β-Hydroxy-DHEA [nM]	0.217 (0.161, 0.285)	0.358 (0.28, 0.45)	0.078	0.103
Androstenediol [nM]	0.587 (0.465, 0.758)	0.501 (0.405, 0.63)	0.519	0.015
Androstenediol sulfate [nM]	186 (140, 252)	152 (118, 198)	0.498	0.017
5-Androstene-3β,7α,17β-triol [pM]	107 (74.8, 148)	118 (84.5, 159)	0.788	0.003
5-Androstene-3β,7β,17β-triol [pM]	79.1 (49.1, 124)	153 (102, 226)	0.156	0.071
5-Androstene-3β,16α,17β-triol [nM]	**0.929 (0.749, 1.15)**	**2.07 (1.66, 2.59)**	**0.001**	**0.301**
5-Androstene-3β,16α,17β-triol sulfate [nM]	**252 (203, 322)**	**133 (112, 160)**	**0.005**	**0.242**
Progesterone [μM]	**0.468 (0.395, 0.559)**	**0.831 (0.673, 1.05)**	**0.009**	**0.222**
17-Hydroxyprogesterone [nM]	**30 (24.8, 36.4)**	**59.8 (48.6, 74.7)**	**0.003**	**0.27**
17,20α-Dihydroxy-4-pregnene-3-one [nM]	**7.33 (5.87, 9.18)**	**19.1 (15, 24.8)**	**<0.001**	**0.347**
17,20α-Dihydroxy-4-pregnene-3-one, conjugated [nM]	**6.39 (4.94, 8.09)**	**11.2 (9.15, 13.6)**	**0.021**	**0.169**
16α-Hydroxyprogesterone [nM]	**23.3 (19.9, 27.5)**	**52.6 (43.3, 65)**	**<0.001**	**0.406**
20α-Dihydroprogesterone [nM]	**86.8 (74.3, 101)**	**216 (181, 261)**	**<0.001**	**0.487**
20α-Dihydroprogesterone, conjugated [nM]	**29.8 (23.6, 37)**	**66.7 (55.6, 79.6)**	**<0.001**	**0.339**
Androstenedione [nM]	6.28 (5.22, 7.67)	9.24 (7.43, 11.8)	0.089	0.1
Testosterone [nM]	2.05 (1.58, 2.69)	2.63 (2, 3.52)	0.394	0.026
Testosterone, conjugated [nM]	10.6 (7.41, 14.5)	12.8 (9.34, 17)	0.579	0.011
16α-Hydroxytestosterone [nM]	**6.07 (4.98, 7.41)**	**14.4 (11.6, 18)**	**<0.001**	**0.36**
16α-Hydroxytestosterone, conjugated [nM]	**3.07 (2.27, 4.06)**	**6.36 (5, 7.99)**	**0.013**	**0.197**
Epitestosterone, conjugated [nM]	15.3 (11.9, 19.8)	21.3 (16.7, 27.4)	0.22	0.053
5α-Dihydrotestosterone [nM]	0.235 (0.161, 0.34)	0.366 (0.253, 0.523)	0.262	0.045
Estrone [nM]	17.6 (13.7, 22.6)	23.4 (18.4, 30)	0.28	0.04
Estrone sulfate [nM]	**253 (188, 329)**	**455 (367, 554)**	**0.029**	**0.159**
Estradiol [nM]	**71.8 (61.7, 83.6)**	**118 (103, 136)**	**0.003**	**0.272**
Estradiol sulfate [nM]	23.4 (19.2, 27.8)	29.1 (24.8, 33.6)	0.223	0.053
Estriol [nM]	60 (46.1, 76.1)	83.2 (66.8, 102)	0.187	0.059
Estriol sulfate [nM]	287 (216, 376)	275 (209, 356)	0.882	<0.001
5α-Dihydroprogesterone [nM]	**86.4 (68.3, 110)**	**206 (159, 272)**	**0.002**	**0.278**
Allopregnanolone [nM]	**33.9 (26.9, 42.4)**	**67 (54, 83.2)**	**0.006**	**0.23**
Allopregnanolone sulfate [μM]	1.78 (1.39, 2.29)	2.03 (1.58, 2.61)	0.632	0.008
Isopregnanolone [nM]	**10.2 (7.76, 13)**	**56 (45.4, 69.3)**	**<0.001**	**0.646**
Isopregnanolone sulfate [μM]	1.08 (0.806, 1.44)	2 (1.49, 2.74)	0.056	0.125
5β-Dihydroprogesterone [nM]	**1.24 (0.832, 1.77)**	**4.66 (3.54, 6.05)**	**<0.001**	**0.372**
Pregnanolone [nM]	18.6 (15.7, 22.1)	25.3 (21.5, 29.9)	0.094	0.097
Pregnanolone, conjugated [μM]	1.06 (0.842, 1.3)	1.16 (0.936, 1.42)	0.677	0.006
Epipregnanolone [nM]	**0.882 (0.709, 1.09)**	**1.62 (1.33, 1.97)**	**0.009**	**0.214**
Epipregnanolone, conjugated [nM]	**182 (143, 230)**	**357 (279, 460)**	**0.013**	**0.203**
5α,20α-Tetrahydroprogesterone [nM]	**34.8 (27.5, 43.9)**	**112 (89.1, 141)**	**<0.001**	**0.453**
5α,20α-Tetrahydroprogesterone, conjugated [nM]	160 (95.8, 260)	263 (166, 409)	0.329	0.033
5α-Pregnane-3α,20α-diol [nM]	**24.8 (19.5, 31.3)**	**88 (70.4, 111)**	**<0.001**	**0.496**
5α-Pregnane-3α,20α-diol, conjugated [μM]	12.1 (8.86, 16.6)	8.14 (6.04, 11)	0.229	0.05
5α-Pregnane-3β,20α-diol [nM]	**4.01 (3.22, 4.95)**	**21 (17.2, 25.8)**	**<0.001**	**0.682**

Table 2. *Cont.*

Steroid	Pregnancy Type		p	η_p^2
	Singleton	Twin		
5α-Pregnane-3β,20α-diol, conjugated [μM]	7.66 (5.67, 10.3)	9.41 (7.06, 12.6)	0.511	0.015
5β,20α-Tetrahydroprogesterone [nM]	**1.66 (1.39, 1.97)**	**3.63 (3.07, 4.31)**	**<0.001**	**0.397**
5β,20α-Tetrahydroprogesterone, conjugated [nM]	53.3 (33.3, 83.2)	56.2 (36, 86.1)	0.909	<0.001
5β-Pregnane-3α,20α-diol [nM]	**12.4 (10.4, 14.7)**	**26.8 (22.4, 32.4)**	**<0.001**	**0.376**
5β-Pregnane-3α,20α-diol, conjugated [μM]	4.81 (3.81, 5.94)	3.26 (2.52, 4.1)	0.125	0.082
5β-Pregnane-3β,20α-diol [nM]	**0.463 (0.326, 0.653)**	**1.18 (0.843, 1.68)**	**0.014**	**0.19**
5β-Pregnane-3β,20α-diol, conjugated [μM]	1.07 (0.839, 1.35)	0.742 (0.565, 0.95)	0.167	0.065
17-Hydroxyallopregnanolone [nM]	**0.386 (0.286, 0.51)**	**1.59 (1.26, 2)**	**<0.001**	**0.51**
17-Hydroxyallopregnanolone sulfate [nM]	**15.1 (11.9, 19.1)**	**49 (37.5, 65.3)**	**<0.001**	**0.423**
17-Hydroxypregnanolone [nM]	**1.01 (0.861, 1.18)**	**1.51 (1.3, 1.77)**	**0.019**	**0.177**
17-Hydroxypregnanolone, conjugate [nM]	74.4 (61.4, 89.9)	111 (92.6, 133)	0.05	0.126
5α-Pregnane-3α,17,20α-triol [pM]	**100 (76.3, 131)**	**282 (214, 377)**	**0.001**	**0.31**
5α-Pregnane-3α,17,20α-triol, conjugated [nM]	47.6 (34.5, 63.9)	70.8 (53.8, 91.9)	0.196	0.057
5β-Pregnane-3α,17,20α-triol [nM]	4.63 (3.83, 5.57)	6.54 (5.51, 7.74)	0.077	0.104
5β-Pregnane-3α,17,20α-triol, conjugated [nM]	380 (301, 485)	441 (367, 535)	0.518	0.011
5α-Androstane-3,17-dione [nM]	**0.379 (0.306, 0.471)**	**0.635 (0.509, 0.799)**	**0.035**	**0.149**
Androsterone [nM]	0.461 (0.379, 0.566)	0.661 (0.538, 0.828)	0.109	0.086
Androsterone sulfate [nM]	291 (220, 381)	188 (140, 249)	0.15	0.073
Epiandrosterone [nM]	**0.143 (0.107, 0.19)**	**0.553 (0.414, 0.747)**	**<0.001**	**0.417**
Epiandrosterone sulfate [nM]	71.9 (57, 91.3)	63 (51, 78.2)	0.58	0.011
Etiocholanolone [pM]	148 (125, 175)	164 (140, 193)	0.55	0.013
Etiocholanolone sulfate [nM]	22.4 (17.3, 30)	18.3 (14.7, 23.4)	0.451	0.02
Epietiocholanolone sulfate [nM]	3.96 (2.53, 6.22)	3.4 (2.21, 5.24)	0.748	0.004
5α-Androstane-3α,17β-diol [pM]	66.8 (53.7, 84.5)	86.2 (68.3, 112)	0.312	0.037
5α-Androstane-3α,17β-diol, conjugated [nM]	**13.7 (11.3, 16.6)**	**21.3 (18.3, 25.1)**	**0.023**	**0.133**
5α-Androstane-3β,17β-diol, conjugated [nM]	**9.58 (7.44, 12.1)**	**18.8 (15.2, 23.1)**	**0.008**	**0.218**
5β-Androstane-3α,17β-diol, conjugated [nM]	2.46 (1.99, 3.07)	3.13 (2.53, 3.93)	0.302	0.037
5β-Androstane-3β,17β-diol, conjugated [nM]	0.34 (0.24, 0.456)	0.532 (0.415, 0.663)	0.129	0.08
Cortisol [nM]	**808 (686, 970)**	**553 (486, 632)**	**0.023**	**0.16**
Cortisol [nM]	**773 (678, 876)**	**579 (505, 660)**	**0.045**	**0.131**
Cortisone [nM]	177 (157, 200)	185 (165, 208)	0.713	0.005
Corticosterone [nM]	24.8 (19.4, 32)	30.6 (23.7, 39.9)	0.441	0.021
11-Deoxycortisol [nM]	8.78 (5.72, 13.3)	18.2 (12.3, 26.9)	0.097	0.092
21-Deoxycortisol [pM]	100 (64.6, 150)	131 (87.9, 190)	0.535	0.013
11-Deoxycorticosterone [nM]	**0.579 (0.307, 0.939)**	**7.44 (5.72, 9.6)**	**<0.001**	**0.654**
11-Deoxycorticosterone sulfate [nM]	**3.34 (1.8, 5.72)**	**15.6 (10.3, 23.1)**	**0.005**	**0.246**
11β-Hydroxyandrostenedione [nM]	46.7 (37.5, 59.2)	58 (45.7, 75.5)	0.391	0.026
11β-Hydroxytestosterone [nM]	**7.49 (6.4, 8.84)**	**15 (12.2, 19)**	**0.001**	**0.319**
11β-Hydroxytestosterone, conjugated, A [nM]	8.78 (5.27, 14.2)	14.8 (9.79, 22.1)	0.266	0.059
11β-Hydroxyandrosterone [pM]	**162 (131, 201)**	**376 (305, 465)**	**<0.001**	**0.348**
11β-Hydroxyandrosterone sulfate [nM]	8.54 (6.71, 11)	8.76 (6.94, 11.1)	0.923	<0.001
11β-Hydroxyepiandrosterone [pM]	**16.3 (11.7, 22.5)**	**89 (63, 128)**	**<0.001**	**0.462**
11β-Hydroxyepiandrosterone sulfate [nM]	7.13 (4.77, 10.5)	9.52 (6.68, 13.6)	0.473	0.02
11β-Hydroxyetiocholanolone [pM]	232 (166, 332)	128 (93.8, 176)	0.094	0.101
11β-Hydroxyetiocholanolone sulfate [nM]	1.5 (1.04, 2.16)	1.14 (0.798, 1.62)	0.479	0.018

The differences between twin and singleton pregnancies for each steroid were evaluated using a linear model consisting of factors Pregnancy type (Twin vs. Singleton) and Gender (Male vs. Female) adjusted for maternal age and gestational age at labour. Significant differences ($p < 0.05$) are in bold, p. . .p-value, η_p^2. . .effect size (0.01~small, 0.06~medium, >0.14~large).

3. Discussion

Due to the major role of the placenta in the metabolism of steroids, we found it crucial to differentiate foetal blood from arterial and venous. Our research therefore analysed these blood samples separately. Comprehensive analysis in this large set of metabolites was never described in detail as we did.

The literature data regarding steroidogenesis and steroid transport in the fetoplacental unit relevant to our results are explained in detail in review articles [4,5]. Looking in

more detail at the results obtained, we see that unconjugated C21 Δ^5 steroids, and most of their unconjugated Δ^4 and 5α/β-reduced metabolites, as well as some 5α/β-reduced C19 steroids and oestradiol, show higher levels in the umbilical vein and artery of twins and especially in the blood of their mothers (Tables 1–3, S1–S3).

Table 3. Characteristics of studied groups, included patients only.

Characteristic	Pregnancy Type	
	Singleton (Control)	Biamniotic Bichorionic Twin
Number of included patients	19	24
Race	Caucasian (19)	Caucasian (24)
Mean age (at the time of delivery)	36.9 (22, 45)	32 (27, 44)
Parity	0.79 (0, 1)	0.71 (0, 4)
Spontaneous conception	15	17
Conception after IVF methods	4	7
Mean weight before pregnancy (kg)	66.6 (49, 122)	73.9 (53, 112)
Mean weight at the time of delivery (kg)	80.1 (65, 118)	94.2 (70, 117)
Mean height (cm)	165.9 (145, 180)	164 (155, 186)
Gestational age at the time of delivery	38.34 (38 + 1, 41 + 1)	37.99 (35 + 3, 39 + 3)
Newborn male sex	9	30
Newborn female sex	10	18
Mean newborn weight	3391.6 (2780, 4050)	2775.5 (1780, 3570)
Mean newborn length *	49.8 (47, 51)	48.1 (43, 51)

Main characteristics of the study and control group. * Mean newborn length in twin foetuses is measured in head presentation only. For this reason, the table does not contain born in breech presentation, as length is measured several days after delivery.

This is to be expected, given that diamniotic dichorionic twins have two foetal adrenal glands and two placentas, with the foetal adrenal being the primary source of C21 Δ^5 steroids in pregnancy, whereas it is the placental steroid sulphatase (STS) for C21 Δ^4 steroids, which hydrolyses sulphates of C21 Δ^5 steroids, mostly of foetal origin, to their unconjugated counterparts and then converts them to their Δ^4 metabolites via HSD3B1, with progesterone being the main product of these processes. In addition, the placenta converts cholesterol and cholesterol sulphate (mostly of maternal origin) into pregnenolone, which is in turn metabolized into progesterone.

In addition, the placenta converts cholesterol and cholesterol sulphate (again predominantly of foetal origin) to pregnenolone, which is then metabolised again to progesterone and further in the placenta, foetal and maternal compartments to its C21 5α reduced metabolites, where the differences are most pronounced, and predominantly in the foetal liver to its C21 5β reduced metabolites, where the differences are less obvious as 5β-reductase activity is absent in the placenta in contrast to 5α-reductase.

Of the C21 Δ^5 steroids, the most pronounced differences are for 16α-hydroxypregnenolone, where the very active 16α-hydroxylation enzyme CYP3A7 in the foetal liver is also involved.

The somewhat less pronounced differences in the 20α-dihydrometabolites of C21 steroids in umbilical cord blood are influenced by the tendency to convert them to 20-oxo analogues in the placenta–foetal direction, and the more pronounced differences in maternal blood by the opposite tendency in the placenta–mother direction. Both phenomena are explained in detail in our previous review [5].

In the case of testosterone levels, which do not differ significantly between singleton and twin pregnancies in all body fluids studied, our data are consistent with the study by Houghton et al. [42], and there are no differences in the cord blood levels of androstenedione either. For oestradiol, like the authors of this study, we also find higher levels in maternal serum, but in addition, we find this difference in umbilical cord venous blood. For estrone, estriol in maternal serum, we agree with the study by Kuiper et al. reporting higher levels of these steroids in maternal blood in twin pregnancies compared to singleton pregnancies [40]. For cord blood, our results and those of the previous study do not match.

The predominant absence of differences in umbilical cord blood for 17-hydroxymetabolites of C21 steroids may be related to the negligible CYP17A1 activity in the placenta compared with both foetus and mother so that the 2-placenta effect is lost here. However, in mothers of twins, the contributions from both foetuses are already additive and the differences here are mostly significant.

For many free androgens, oestrogens, 11-deoxycorticoids, 11β-hydroxy androgens, and corticosterone, there is again a clear trend towards higher levels in twins, and especially in their mothers. Exceptions are conjugated 11β-hydroxytestosterone with lower levels in umbilical arterial blood, cortisol, and cortisone, whose levels are lower in the blood of mothers of twins compared with mothers of singleton pregnancies.

Compared to unconjugated steroids, with a tendency towards higher levels in twins and especially in their mothers, conjugated steroids show the opposite trend, with lower levels in twins and, again, especially in their mothers. This phenomenon can be explained by the influence of two placentas and thus higher overall placental STS activity, leading to lower levels of conjugated steroids but a further increase in levels of their free analogues in twin pregnancies. This trend is very pronounced not only for the C21 Δ^5 steroid pregnenolone sulphate but also for the C19 Δ^5 steroid DHEA sulphate, and sometimes occurs with other steroid conjugates.

Finally, the results of the OPLS/OMR analysis show effective discrimination between singleton and twin pregnancies for arterial cord blood (sensitivity = 0.962 (0.888–1, shown as mean with 95% confidence interval), specificity = 1 (1–1)) (Table S1), good discrimination for venous cord blood (sensitivity = 0.943 (0.866–1), specificity = 0.8 (0.598–1)) (Table S2), and absolute discrimination for maternal blood (sensitivity = 1 (1–1), specificity = 1 (1–1)) (Table S3). The results show that the overall doubling effect of foetal and placental steroidogenic tissues is most evident in maternal blood, although the levels of many steroids in maternal blood are significantly lower compared to cord blood.

Different levels of steroids (which are often bioactive) in singleton and twin pregnancies can influence a number of physiological and pathophysiological processes. Steroids act on a number of nuclear and ionotropic receptors, may positively or negatively influence the activity of detoxifying enzymes [43], and as a result may influence a number of pregnancy pathologies such as intrahepatic cholestasis of pregnancy [14], gestational diabetes [44], preeclampsia [17–19], foetal growth restriction [20], acute hypoxia [21–23], and postpartum depression [24,25]. The coincidence between depression and allopregnanolone levels can be observed in the third trimester and later [26], altered pain transmission and perception [27,43], altered gestational length [5], altered immune response [43,45], and possibly also programming of individual development in childhood and adulthood [46].

Regarding sex differences, our data show elevated levels of testosterone, 5α-dihydrotestosterone, and 5-androstene-3β,16α,17β-triol sulphate (which is a metabolite of one of the testosterone precursors androstenediol), with decreased levels of the other testosterone precursor androstenedione in arterial and venous umbilical cord blood (Tables S4 and S5), which may be related to steroidogenesis in the testes of male foetuses. Conversely, the interpretation of reduced levels of other steroids in the umbilical cord blood of male foetuses and significant differences in maternal venous blood remains (Table S6) an open question for further research beyond the scope of this study. The literature reports the independence of key maternal blood steroids from foetal sex [37], and our data show differences for some steroids. However, interpretation of these results also requires further research beyond the scope of this study.

In discussing the differences between analytes in different body fluids, we found less obvious differences between arterial and venous blood. However, there were mostly very substantial differences between venous and maternal blood, which is consistent with our previous studies.

The steroid metabolome is closely linked to the timing of labour and affects several pregnancy pathologies. These components are well described in singleton pregnancies.

A large set of steroid metabolome components, such as these, are within the multiple pregnancies described for the first time.

Because of the different pathways of steroid hormone synthesis, we differentiated between male and female foetuses and found some differences between the sexes that can be partly explained by testicular steroidogenesis in male foetuses. Some negative correlations of steroid levels with gestational age may be related to planning for a caesarean section. Most surgeries are performed before the calculated due date. The same results were observed in the control group (47 foetuses).

Because we excluded women with any issues that could have affected the results, our results showed no association with pregnancy pathologies. This should be the focus of a following study. Throughout the study, babies were delivered exclusively by planned caesarean section. The placental "timing" function had been eliminated. Therefore, our results did not show any changes in the steroidome related to the onset of labour. In addition, gestational age was included in the model as one of the covariates.

Our study may have several limitations we had to work with. Variation of gestational age is influenced by the date the pregnancy was terminated. This is managed by national guidelines for twin pregnancy care. In some cases of smaller foetuses, we were limited by the sample size—umbilical cord vessels are significantly thinner. Blood in these vessels clots almost immediately after cutting the umbilical cord. For proper analysis, at least 3 mL of blood is needed. In case of insufficient blood draw, analysis is almost impossible.

Investigation of dichorionic diamniotic twins raises a genetic issue; there may be cases in which embryos split in a very early phase, resulting in monozygotic twins with identical genetic information. Thus, the role of genetic influences, particularly in terms of enzymatic activity, can only be discussed theoretically.

Research and statistical analysis show that there are significant differences between singleton and dichorionic diamniotic twin pregnancies. Differences are based on the fact that there are two foetuses. Compared with singleton pregnancies, the foetal contribution can be considered as doubled in twins. Higher enzymatic activity is caused by two adrenal glands and two separate placentae.

This study demonstrates the scope for further research on multiple pregnancies. The next step should be the analysis of the steroid metabolome in multiple pregnancies with spontaneous onset of labour, in pathologies such as preeclampsia, or when signs of spontaneous premature labour are present. Further analysis based on this study can help to understand the pathophysiology of the pathologies mentioned above.

4. Methods and Materials

4.1. Study Participants

This study included two groups of patients: pregnant women with multiple pregnancies as the study group, and singleton pregnancies as the control group. Patient enrolment was conducted from November 2019 to November 2021.

All patients aged 18 years and above who met the inclusion criteria were asked to participate in the study. The eligibility criteria were twin pregnancy (for the study group), planned delivery to the author's obstetrics clinic, and planned caesarean section due to multiple pregnancies. Dichorionic diamniotic pregnancies were terminated in the 34th–39th week of pregnancy.

We excluded women with metabolic disorders that could influence steroid metabolism, especially diabetes mellitus and intrahepatic cholestasis during pregnancy, women with intake of progesterone during pregnancy, women with acute malignant process, women with serious pathologies affecting pregnancy (e.g., twin-to-twin transfusion syndrome), women who were administered corticosteroids due to foetal lung maturation, smokers, and those who did not consent to participation in a study. We did not exclude IVF-conceived pregnancies.

The control group comprised women with singleton pregnancies. These patients were scheduled for caesarean section due to other medical conditions (e.g., caesarean section

in the medical history, another type of uterine scar, breech presentation). The exclusion criteria were the same as for twin pregnancies (e.g., acute malignancy, serious metabolic disorder, disagreement).

Both groups included women after negative results of combined first-trimester screening and second-trimester morphology scan.

Blood samples were collected from 50 women with twin pregnancies and 20 with singleton pregnancies in the control group.

4.2. Specimen Collection and Pre-Laboratory Processing

Every consenting patient had venous maternal blood drawn at the latest possible time before the caesarean section and before administering any medication. Immediately after birth, arterial and venous blood were collected separately from each twin's umbilical cord. We used the part of the cord attached to the placenta; therefore, the newborn was unaffected. For purposes of the research, we used the BDVacutainer® K2EDTA Vacutainer system (Becton Dickinson, Franklin Lakes, NJ, USA). Blood volume was 6 mL from the mother and 3 mL from the umbilical artery and veins.

Samples were processed no longer than 2 h after collection. After clotting at room temperature, the sample was centrifuged for 10 min at 2400 rpm. The serum was then transferred to a microtube and frozen at −18 °C. The frozen samples were transported to the laboratory in an icebox. Several studies in the past showed that samples taken and frozen using this technique are very stable, and the concentrations of steroids do not change over time [47,48].

The same procedure was used for both the study group and the control group.

4.3. Laboratory Processing

All laboratory measurements were performed at the Department of Steroids and Proteofactors, Institute of Endocrinology, Prague, Czech Republic. Gas chromatography-tandem mass spectrometry (GC-MS/MS) was used for the sample analysis. The equipment included a gas chromatograph with automatic flow control, an autosampler, and a triple quadrupole detector with an adjustable electron voltage. The measurement is based on ionisation after electron impact. Hill developed, described, and published the method. The list of analysed metabolites is based on this study (see Supplementary Table S7) [49].

4.4. Clinical Data

Information about the mother, foetus, delivery, and newborn was extracted from medical records. The basic characteristic is provided in Table 3.

The information included the medical history of the pregnant women, age, education, race, general medical history, information about smoking and alcohol/drugs abuse, weight before and after pregnancy, height, parity, method of conception, biochemical and ultrasound markers of the foetus throughout the pregnancy, information about any problems during pregnancy, biochemical and blood test results from pregnancy (if provided by a gynaecologist providing regular checks during pregnancy), medication taken during pregnancy, vitals obtained during caesarean section, foetal sex, weight and length, APGAR score, pH and Astrup values, and information about postpartal adaptation.

4.5. Data Evaluation

In the first step, the power transformation parameters were found for each metric variable so that its distribution was as close as possible to the Gaussian distribution. The differences between twin and singleton pregnancies for each steroid were evaluated using a linear model consisting of factors Pregnancy type (Twin vs. Singleton) and Gender (Male vs. Female), adjusted for maternal age and gestational age at labour. The statistical software Statgraphics Centurion v. XVIII from Statgraphics Technologies, Inc. (The Plains, VA, USA) was used for the above analyses.

In addition, the differences between twin and singleton pregnancies (parameter status, twin vs. singleton pregnancy) were simultaneously tested for all steroids, maternal age and gestational age at labour in maternal, umbilical arterial, and umbilical venous blood, using multivariate regression models with a reduction in dimensionality known as orthogonal predictions to latent structure (OPLS) and ordinary multiple regression (OMR) [14,43].

Flow chart of OPLS analysis is added as Supplement Table S8. Power calculation for the study is included in Supplement Table S9.

The OPLS model, which is a multivariate regression with dimensionality reduction, allows for the evaluation of relationships between explained variables and the explaining variables (predictors) that may be highly correlated, which is also the case for steroids in metabolic pathways. The presence of the twin pregnancy in the OPLS model is expressed as the logarithm of the likelihood ratio (the ratio of the probability of the presence of twin pregnancy p to the probability of singleton pregnancy $(1 - p)$), i.e., the logarithm of the likelihood ratio is calculated, which then ranges from -infinity to + infinity. This approach ensures that the prediction of the probability of the presence of pathology is between 0 and 1 (after applying a recurrent formula that converts the prediction of the logarithm of likelihood ratio into a prediction of the probability of the presence of pathology).

The variability in the predictors is divided into two independent components. The first contains the variability of predictors that were shared with the probability of twin pregnancy (predictive component), whereas the orthogonal components explained the variability shared within highly correlated predictors. OPLS identifies relevant predictors, as well as the best linear combination of predictors to estimate the probability of the presence of pathology. After standardization of the variables, the OPLS model can be expressed as follows:

$$X = T_p P_p^T + T_0 P_0^T + E \quad (1)$$

$$Y = T_p P_p^T + F \quad (2)$$

where X is the matrix with predictors and subjects; Y is the vector of dependent variable and subjects; T_p is the vector of component scores from the single predictive component and subjects extracted from Y; T_o is the vector of component scores from the single orthogonal component and subjects extracted from X; P_p is the vector of component loadings for the predictive component extracted from Y; P_o is the vector of component loadings for the orthogonal component extracted from X and independent variables; and E and F are the error terms.

The relevant predictors were chosen using variable importance (VIP) statistics. The statistical software SIMCA-P v.12.0 from Umetrics AB (Umeå, Sweden), which was used for OPLS analysis, enabled the finding of the number of relevant components, the detection of multivariate non-homogeneities, and testing the multivariate normal distribution and homoscedasticity (constant variance).

The algorithm for obtaining the predictions was as follows:

- Transformation of the original data to obtain the values with symmetric distribution and constant variance
- Checking the data homogeneity in predictors using Hotelling's statistics and the eventual elimination of non-homogeneities
- Testing the relevance of predictors using variable importance statistics and the elimination of irrelevant predictors
- Calculating component loadings for individual variables to evaluate their correlations with the predictive component
- Calculating regression coefficients for the multiple regression model to evaluate the mutual independence of predictors after comparison with the corresponding component loadings from the OPLS model
- Calculating predicted values of the logarithm of the ratio of the probability of twin pregnancy presence to the probability of singleton pregnancy (LLR)
- Calculating the probability of the twin pregnancy presence for individual subjects

- Calculating the sensitivity and specificity of the prediction

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25031591/s1.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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References

1. Rupprecht, R.; Reul, J.M.; Trapp, T.; van Steensel, B.; Wetzel, C.; Damm, K.; Zieglgänsberger, W.; Holsboer, F. Progesterone receptor-mediated effects of neuroactive steroids. *Neuron* **1993**, *11*, 523–530. [CrossRef] [PubMed]
2. Tuem, K.B.; Atey, T.M. Neuroactive steroids: Receptor interactions and responses. *Front. Neurol.* **2017**, *8*, 442. [CrossRef] [PubMed]
3. Mitchell, B.F.; Mitchell, J.M.; Chowdhury, J.; Tougas, M.; Engelen, S.M.; Senff, N.; Heijnen, I.; Moore, J.T.; Goodwin, B.; Wong, S.; et al. Metabolites of progesterone and the pregnane X receptor: A novel pathway regulating uterine contractility in pregnancy? *Am. J. Obstet. Gynecol.* **2005**, *192*, 1304–1315. [CrossRef] [PubMed]
4. Pasqualini, J.R.; Chetrite, G.S. The formation and transformation of hormones in maternal, placental and fetal compartments: Biological implications. *Horm. Mol. Biol. Clin. Investig.* **2016**, *27*, 11–28. [CrossRef] [PubMed]
5. Pašková, A. Úloha neuroaktivních steroidů v těhotenství a jejich význam v prenatální diagnostice. [The role of neuroactive steroids in pregnancy and their importance in prenatal diagnostics]. *Praha* **2013**, *100*, 3.
6. Adamcová, K.; Kolátorová, L.; Škodová, T.; Šimková, M.; Pařízek, A.; Stárka, L.; Dušková, M. Steroid hormone levels in the peripartum period—Differences caused by fetal sex and delivery type. *Physiol. Res.* **2018**, *67*, S489–S497. [CrossRef] [PubMed]
7. Ellis, M.J.; Livesey, J.H.; Inder, W.J.; Prickett, T.C.; Reid, R. Plasma corticotropin-releasing hormone and unconjugated estriol in human pregnancy: Gestational patterns and ability to predict preterm delivery. *Am. J. Obstet. Gynecol.* **2002**, *186*, 94–99. [CrossRef]
8. Hill, M.; Parízek, A.; Kancheva, R.; Dusková, M.; Velíková, M.; Kríz, L.; Klímková, M.; Pasková, A.; Zizka, Z.; Matucha, P.; et al. Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and in amniotic fluid in normal and preterm labor. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 594–610. [CrossRef]
9. McLean, M.; Smith, R. Corticotrophin-releasing hormone and human parturition. *Reproduction* **2001**, *121*, 493–501. [CrossRef]
10. Ravanos, K.; Dagklis, T.; Petousis, S.; Margioula-Siarkou, C.; Prapas, Y.; Prapas, N. Factors implicated in the initiation of human parturition in term and preterm labor: A review. *Gynecol. Endocrinol.* **2015**, *31*, 679–683. [CrossRef]
11. Linton, E.A.; Woodman, J.R.; Asboth, G.; Glynn, B.P.; Plested, C.P.; Bernal, A.L. Corticotrophin releasing hormone: Its potential for a role in human myometrium. *Exp. Physiol.* **2001**, *86*, 273–281. [CrossRef]
12. Grammatopoulos, D.; Thompson, S.; Hillhouse, E.W. The human myometrium expresses multiple isoforms of the corticotropin-releasing hormone receptor. *J. Clin. Endocrinol. Metab.* **1995**, *80*, 2388–2393. [PubMed]
13. Zhu, P.; Tao, F.B.; Jiang, X.M.; Hao, J.H.; Wang, Y.L.; Xu, Y.Y. Effect of intrahepatic cholestasis of pregnancy on the functions of hypothalamic-pituitary-adrenocortical axis and adrenal cortex in normal neonates. *Chin. J. Contemp. Pediatr.* **2010**, *12*, 5–8.
14. Pařízek, A.; Hill, M.; Dušková, M.; Vítek, L.; Velíková, M.; Kancheva, R.; Šimják, P.; Koucký, M.; Kokrdová, Z.; Adamcová, K.; et al. A comprehensive evaluation of steroid metabolism in women with intrahepatic cholestasis of pregnancy. *PLoS ONE* **2016**, *11*, e0159203. [CrossRef] [PubMed]
15. Šimják, P.; Pařízek, A.; Vítek, L.; Černý, A.; Adamcová, K.; Koucký, M.; Hill, M.; Dušková, M.; Stárka, L. Fetal complications due to intrahepatic cholestasis of pregnancy. *J. Perinat. Med.* **2015**, *43*, 133–139. [CrossRef] [PubMed]

16. Pařízek, A.; Dušková, M.; Vítek, L.; Šrámková, M.; Hill, M.; Adamcová, K.; Šimják, P.; Černý, A.; Kordová, Z.; Vráblíková, H.; et al. The role of steroid hormones in the development of intrahepatic cholestasis of pregnancy. *Physiol. Res.* **2015**, *64*, S203–S209. [CrossRef] [PubMed]
17. Luisi, S.; Petraglia, F.; Benedetto, C.; Nappi, R.E.; Bernardi, F.; Fadalti, M.; Reis, F.M.; Luisi, M.; Genazzani, A.R. Serum allopregnanolone levels in pregnant women: Changes during pregnancy, at delivery, and in hypertensive patients. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 2429–2433. [CrossRef] [PubMed]
18. Parker, C.R., Jr.; Everett, R.B.; Quirk, J.G., Jr.; Whalley, P.J.; Gant, N.F. Hormone production during pregnancy in the primigravid patient. I. Plasma levels of progesterone and 5-alpha-pregnane-3,20-dione throughout pregnancy of normal women and women who developed pregnancy-induced hypertension. *Am. J. Obstet. Gynecol.* **1979**, *135*, 778–782. [CrossRef] [PubMed]
19. Berkane, N.; Liere, P.; Oudinet, J.P.; Hertig, A.; Lefèvre, G.; Pluchino, N.; Schumacher, M.; Chabbert-Buffet, N. From pregnancy to preeclampsia: A key role for estrogens. *Endocr. Rev.* **2017**, *38*, 123–144. [CrossRef]
20. Baud, O.; Berkane, N. Hormonal changes associated with intra-uterine growth restriction: Impact on the developing brain and future neurodevelopment. *Front. Endocrinol.* **2019**, *10*, 179. [CrossRef]
21. Hirst, J.J.; Yawno, T.; Nguyen, P.; Walker, D.W. Stress in pregnancy activates neurosteroid production in the fetal brain. *Neuroendocrinology* **2006**, *84*, 264–274. [CrossRef]
22. Hirst, J.J.; Palliser, H.K.; Yates, D.M.; Yawno, T.; Walker, D.W. Neurosteroids in the fetus and neonate: Potential protective role in compromised pregnancies. *Neurochem. Int.* **2008**, *52*, 602–610. [CrossRef]
23. Hirst, J.J.; Kelleher, M.A.; Walker, D.W.; Palliser, H.K. Neuroactive steroids in pregnancy: Key regulatory and protective roles in the foetal brain. *J. Steroid Biochem. Mol. Biol.* **2014**, *139*, 144–153. [CrossRef]
24. McEvoy, K.; Payne, J.L.; Osborne, L.M. Neuroactive steroids and perinatal depression: A review of recent literature. *Curr. Psychiatry Rep.* **2018**, *20*, 78. [CrossRef] [PubMed]
25. Deligiannidis, K.M.; Kroll-Desrosiers, A.R.; Mo, S.; Nguyen, H.P.; Svenson, A.; Jaitly, N.; Hall, J.E.; Barton, B.A.; Rothschild, A.J.; Shaffer, S.A. Peripartum neuroactive steroid and γ-aminobutyric acid profiles in women at-risk for postpartum depression. *Psychoneuroendocrinology* **2016**, *70*, 98–107. [CrossRef] [PubMed]
26. Hellgren, C.; Comasco, E.; Skalkidou, A.; Sundström-Poromaa, I. Allopregnanolone levels and depressive symptoms during pregnancy in relation to single nucleotide polymorphisms in the allopregnanolone synthesis pathway. *Horm. Behav.* **2017**, *94*, 106–113. [CrossRef] [PubMed]
27. Todorovic, S.M.; Pathirathna, S.; Brimelow, B.C.; Jagodic, M.M.; Ko, S.H.; Jiang, X.; Nilsson, K.R.; Zorumski, C.F.; Covey, D.F.; Jevtovic-Todorovic, V. 5beta-reduced neuroactive steroids are novel voltage-dependent blockers of T-type Ca2+ channels in rat sensory neurons in vitro and potent peripheral analgesics in vivo. *Mol. Pharmacol.* **2004**, *66*, 1223–1235. [CrossRef] [PubMed]
28. Zorumski, C.F.; Paul, S.M.; Covey, D.F.; Mennerick, S. Neurosteroids as novel antidepressants and anxiolytics: GABA-A receptors and beyond. *Neurobiol. Stress* **2019**, *11*, 100196. [CrossRef] [PubMed]
29. Anker, J.J.; Carroll, M.E. The role of progestins in the behavioral effects of cocaine and other drugs of abuse: Human and animal research. *Neurosci. Biobehav. Rev.* **2010**, *35*, 315–333. [CrossRef] [PubMed]
30. Good Clinical Practice Network. A Study of Brexanolone for Acute Respiratory Distress Syndrome Due to COVID-19. Available online: https://ichgcp.net/clinical-trials-registry/NCT04537806 (accessed on 29 March 2022).
31. Shaw, J.C.; Dyson, R.M.; Palliser, H.K.; Gray, C.; Berry, M.J.; Hirst, J.J. Neurosteroid replacement therapy using the allopregnanolone-analogue ganaxolone following preterm birth in male guinea pigs. *Pediatr. Res.* **2019**, *85*, 86–96. [CrossRef]
32. Chauhan, S.P.; Scardo, J.A.; Hayes, E.; Abuhamad, A.Z.; Berghella, V. Twins: Prevalence, problems, and preterm births. *Am. J. Obstet. Gynecol.* **2010**, *203*, 305–315. [CrossRef]
33. Sentilhes, L.; Oppenheimer, A.; Bouhours, A.C.; Normand, E.; Haddad, B.; Descamps, P.; Marpeau, L.; Goffinet, F.; Kayem, G. Neonatal outcome of very preterm twins: Policy of planned vaginal or cesarean delivery. *Am. J. Obstet. Gynecol.* **2015**, *213*, 73.e1–73.e7. [CrossRef]
34. Townsend, R.; Khalil, A. Fetal growth restriction in twins. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *49*, 79–88. [CrossRef]
35. Bamberg, C.; Hecher, K. Update on twin-to-twin transfusion syndrome. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2019**, *58*, 55–65. [CrossRef]
36. Canpolat, F.E.; Yurdakök, M.; Korkmaz, A.; Yigit, S.; Tekinalp, G. Birthweight discordance in twins and the risk of being heavier for respiratory distress syndrome. *Twin Res. Hum. Genet.* **2006**, *9*, 659–663. [CrossRef] [PubMed]
37. Cohen-Bendahan, C.C.; van Goozen, S.H.; Buitelaar, J.K.; Cohen-Kettenis, P.T. Maternal serum steroid levels are unrelated to fetal sex: A study in twin pregnancies. *Twin Res. Hum. Genet.* **2005**, *8*, 173–177. [CrossRef] [PubMed]
38. Tapp, A.L.; Maybery, M.T.; Whitehouse, A.J. Evaluating the twin testosterone transfer hypothesis: A review of the empirical evidence. *Horm. Behav.* **2011**, *60*, 713–722. [CrossRef] [PubMed]
39. Talia, C.; Raja, E.A.; Bhattacharya, S.; Fowler, P.A. Testing the twin testosterone transfer hypothesis-intergenerational analysis of 317 dizygotic twins born in Aberdeen, Scotland. *Hum. Reprod.* **2020**, *35*, 1702–1710. [CrossRef] [PubMed]
40. Kuijper, E.A.; Twisk, J.W.; Korsen, T.; Caanen, M.R.; Kushnir, M.M.; Rockwood, A.L.; Meikle, A.W.; Hompes, P.G.; Wit, J.M.; Lambalk, C.B. Mid-pregnancy, perinatal, and neonatal reproductive endocrinology: A prospective cohort study in twins and singleton control subjects. *Fertil. Steril.* **2015**, *104*, 1527–1534.e349. [CrossRef] [PubMed]
41. Thomas, H.V.; Murphy, M.F.; Key, T.J.; Fentiman, I.S.; Allen, D.S.; Kinlen, L.J. Pregnancy and menstrual hormone levels in mothers of twins compared to mothers of singletons. *Ann. Hum. Biol.* **1998**, *25*, 69–75. [CrossRef] [PubMed]

42. Houghton, L.C.; Lauria, M.; Maas, P.; Stanczyk, F.Z.; Hoover, R.N.; Troisi, R. Circulating maternal and umbilical cord steroid hormone and insulin-like growth factor concentrations in twin and singleton pregnancies. *J. Dev. Orig. Health Dis.* **2019**, *10*, 232–236. [CrossRef] [PubMed]
43. Honcu, P.; Hill, M.; Bicikova, M.; Jandova, D.; Velikova, M.; Kajzar, J.; Kolatorova, L.; Bestak, J.; Macova, L.; Kancheva, R.; et al. Activation of Adrenal Steroidogenesis and an Improvement of Mood Balance in Postmenopausal Females after Spa Treatment Based on Physical Activity. *Int. J. Mol. Sci.* **2019**, *20*, 3687. [CrossRef] [PubMed]
44. Shin, Y.Y.; An, S.M.; Jeong, J.S.; Yang, S.Y.; Lee, G.S.; Hong, E.J.; Jeung, E.B.; Kim, S.C.; An, B.S. Comparison of steroid hormones in three different preeclamptic models. *Mol. Med. Rep.* **2021**, *23*, 252. [CrossRef] [PubMed]
45. Solano, M.E.; Arck, P.C. Steroids, Pregnancy and Fetal Development. *Front. Immunol.* **2019**, *10*, 3017. [CrossRef] [PubMed]
46. Vo, T.; Hardy, D.B. Molecular mechanisms underlying the fetal programming of adult disease. *J. Cell Commun. Signal.* **2012**, *6*, 139–153. [CrossRef] [PubMed]
47. Kley, H.K.; Schlaghecke, R.; Krüskemper, H.L. Stabilität von Steroiden im Plasma über einen Zeitraum von 10 Jahren [Stability of steroids in plasma over a 10-year period]. *J. Clin. Chem. Clin. Biochem.* **1985**, *23*, 875–878.
48. Kley, H.K.; Rick, W. Einfluss von Lagerung und Temperatur auf die Analyse von Steroiden in Plasma und Blut [The effect of storage and temperature on the analysis of steroids in plasma and blood]. *J. Clin. Chem. Clin. Biochem.* **1984**, *22*, 371–378.
49. Hill, M.; Hána, V., Jr.; Velíková, M.; Pařízek, A.; Kolátorová, L.; Vítků, J.; Škodová, T.; Šimková, M.; Šimják, P.; Kancheva, R.; et al. A method for determination of one hundred endogenous steroids in human serum by gas chromatography-tandem mass spectrometry. *Physiol. Res.* **2019**, *68*, 179–207. [CrossRef]

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Steroidní metabolom a vícečetné těhotenství

Steroid metabolome and multiple pregnancy

A. Černý[1], Z. Laštůvka[1], M. Hill[2], A. Pařízek[1]

[1] Gynekologicko-porodnická klinika 1. LF UK a VFN v Praze
[2] Oddělení steroidů a proteofaktorů, Endokrinologický ústav v Praze

Souhrn: V závislosti na místě jejich aktivity mohou být steroidy rozděleny do dvou skupin – intracelulární a extracelulární. Intracelulární působí jako transkripční faktory, potlačují nebo aktivují expresi genů – mají tzv. genomový efekt, a proto je jejich nástup účinku pomalý. Steroidy působící extracelulárně (negenomový účinek) se vážou na neurotransmiterové receptory umístěné na cytoplazmatické buněčné membráně, a ovlivňují tak propustnost iontových kanálů, přičemž jejich účinek je mnohem rychlejší a označujeme je jako neuroaktivní steroidy či neurosteroidy. Zatímco neuroaktivní steroidy mohou být produkovány v různých tkáních organizmu nebo mohou být aplikovány zvnějšku, neurosteroidy jsou syntetizovány v buňkách nervového systému. Některé neuroaktivní steroidy, jejichž hladiny jsou v těhotenství extrémně zvýšeny (progesteron a jeho metabolity), mají zásadní význam ve stabilizaci těhotenství a změny jejich koncentrace mohou ovlivňovat mechanizmus začátku porodu. Poruchy steroidogeneze se mohou podílet na vzniku celé řady těhotenských patologií, jako jsou předčasný porod, preeklampsie, intrahepatální cholestáza v těhotenství apod. Náš výzkum ve spolupráci s Oddělením steroidů a proteofaktorů Endokrinologického ústavu v Praze se zaměřuje také na zkoumání vícečetných těhotenství z hlediska biosyntézy, transportu a účinku steroidů. Dosavadní studie dostupné v literatuře zatím neposkytly ucelenou analýzu steroidomu u dětí a matek u vícečetného těhotenství. Cílem našeho výzkumu je proto objasnit vztahy mezi plody a matkou a mezi plody vzájemně z hlediska syntézy a transportu steroidů i z hlediska fyziologie a patofyziologie lidského těhotenství a porodu.

Klíčová slova: steroidní metabolom – neuroaktivní steroidy – těhotenské komplikace – vícečetné těhotenství

Summary: Steroid biosynthesis occurs in adrenal, gonadal, brain, liver, and placental tissues. Depending on the location of their activity, steroids can be divided into two groups – intracellular and extracellular. Intracellular ones act as transcription factors, suppressing or activating gene expression – they have a so-called genomic effect and therefore their onset of action is slow. Steroids acting extracellularly (non-genome effect) bind to neurotransmitter receptors located on the cytoplasmic cell membrane and thus affect the permeability of the ion channels, the effect of which is much faster, and we refer to them as neuroactive steroids or neurosteroids. While neuroactive steroids can be produced in different tissues of the body, or can be administered externally, neurosteroids are synthetized in cells of the nervous system. Some neuroactive steroids whose levels are extremely elevated in pregnancy (progesterone and its metabolites) are crucial in stabilizing pregnancy and changes in their concentration may influence the onset of parturition. Steroidogenic disorders may be involved in a number of pregnancy pathologies such as premature birth, pre-eclampsia, intrahepatic cholestasis in pregnancy, etc. Our research in collaboration with the Department of Steroids and Proteofactors of the Institute of Endocrinology in Prague also focuses on the investigation of multiple pregnancies in terms of biosynthesis, transport, and the effects of steroids. Studies available in the literature so far have not provided a comprehensive analysis of the steroidome in children and mothers in multiple pregnancies. The aim of our research is therefore to clarify the relationships between fetuses and mothers and between fetuses from the point of view of steroid synthesis and transport as well as the physiology and pathophysiology of human pregnancy and childbirth.

Key words: fetomaternal steroidome – neuroactive steroids – pregnancy complication – multiple pregnancy

Úvod

Biosyntéza steroidních hormonů probíhá ve tkáních nadledvin, gonád, mozku, jater a v placentě.

Prekurzorem pro jejich syntézu je cholesterol a jeho sulfát. Zjednodušené schéma syntézy steroidů popisuje schéma 1 [1]. Souhrn všech metabolitů steroidů označujeme jako steroidní metabolom či zkráceně steroidom.

Mechanizmus účinku steroidů je trojí:

1. Genomový „klasický" – po vazbě na intracelulární receptory nebo přímo na DNA. Působí jako transkripční faktory, aktivují nebo potlačují expresi genů. Výsledkem je syntéza bílkovin. Efekt se projeví obvykle až za několik hodin nebo dní.
2. Negenomový – steroidy se vážou na neurotransmiterové receptory (iontové kanály) lokalizované na cytoplaz-

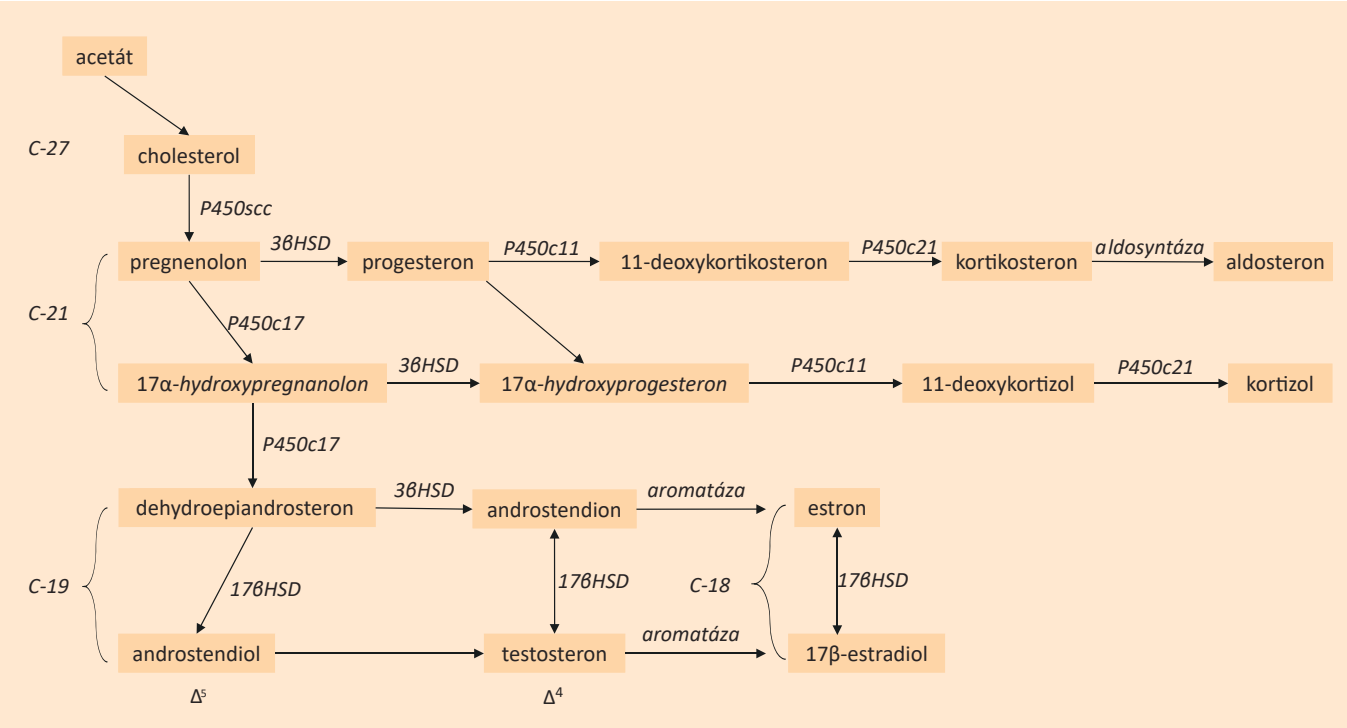


Schéma 1. Schéma syntézy steroidů, převzato ze Strauss a Barbieri [1].
Scheme 1. Scheme of steroid synthesis, taken from Strauss and Barbieri [1].

matické membráně, a tím ovlivňují neuronální excitabilitu. Nástup účinku je velmi rychlý, projevuje se za řádově sekundy až milisekundy. Negenomovým účinkem působí tzv. neuroaktivní steroidy vč. neurosteroidů (ty jsou syntetizovány v nervovém systému).

3. V mozku některé steroidy, a to jak neurosteroidy pronikající do mozku z cirkulace přes hematoencefalickou bariéru, tak neurosteroidy v mozku syntetizované, působí negenomovým účinkem vazbou na membránové receptory [2,3].

Neurosteroidy jsou steroidní hormony syntetizované ve tkáních nervového systému. Neuroaktivní steroidy jsou všechny steroidy s neuroaktivním účinkem bez ohledu na místo jejich syntézy.

Z neuroaktivních steroidů mají v těhotenství zejména význam progesteron a řada jeho metabolitů (tzv. neuroaktivní steroidy pregnanového typu) – allopregnanolon, isopregnanolon, pregnanolon a epipregnanolon. Do skupiny neuroaktivních steroidů mohou patřit estrogeny, které vedle toho působí i genomově, část nadledvinových androgenů, zatímco kortikosteroidy působí pouze genomově.

Neuroaktivní steroidy se vážou na receptory typu A kyseliny γ-aminomáselné (GABAA-r) a N-methyl-D-aspartátové receptory (NMDA-r). Vazbou na GABAA-r se uvolňují chloridové kanály, zatímco vazbou na NDMA-r kanály kalciové. Kromě centrálního nervového systému (CNS – central nervous system) se receptory pro neuroaktivní steroidy nacházejí také na děloze, čímž se např. vysvětluje možnost ovlivnění děložní kontraktility [4].

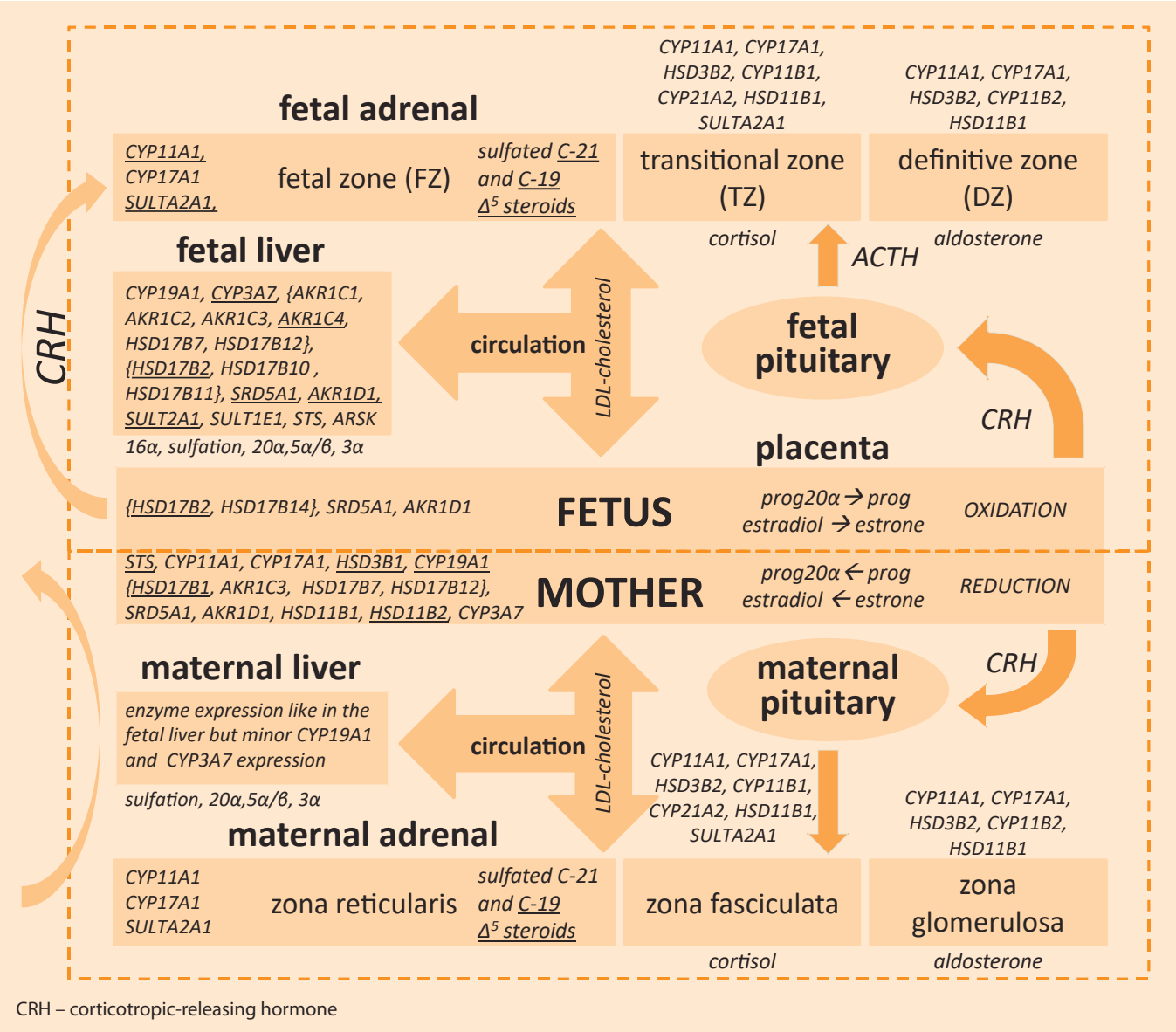
V průběhu těhotenství je endokrinní aktivita fetoplacentární jednotky velmi vysoká. U plodu je primárním místem syntézy neuroaktivních steroidů tzv. fetální zóna fetální nadledviny. Aktivita této zóny je stimulována produkcí placentárního kortikotropního hormonu (CRH – corticotropic-releasing hormone). Vzhledem k absenci některých enzymů v placentě nebo naopak u plodu je nutný transport hormonů a jejich prekurzorů v rámci fetoplacentární jednotky. Cílem je syntéza dostatečného množství steroidních hormonů pro vývoj plodu [5].

V rámci našich předchozích studií bylo navrženo schéma steroidogeneze v těhotenství (schéma 2) [6].

Analýza steroidomu

Ke stanovení se kromě rutinních imunoanalytických metod využívají metody chromatografické, jako je plynová chromatografie s hmotnostní detekcí [7] či kapalinová chromatografie s tandemovou hmotnostní detekcí. Podle některých autorů je tato metoda vhodnější ke stanovení metabolitů kortikosteroidů [8,9]. Existují i studie, které využívají tuto metodu k analýze steroidního metabolomu u fertilních nebo těhotných žen [10,11].

Pro komplexní kvantifikaci steroidomu u těhotných žen a plodů byly také vyvinuty metody plynové chromatografie s tandemovou hmotnostní detekcí.



CRH – corticotropic-releasing hormone

Schéma 2. Zjednodušené schéma steroidogenezy ve fetoplacentární jednotce [6].
Scheme 2. Simplified scheme of steroidogenesis in the fetoplacental unit [6].

Tyto metody nabízejí možnost simultánní kvantifikace až 100 endogenních steroidů. Kromě analýzy steroidomu v pupečníkové krvi byl tento postup nedávno validován i pro analýzu v krvi těhotných žen a dále v krvi netěhotných žen v obou fázích menstruačního cyklu a v krvi mužů [12].

Klinický význam stanovení a studia steroidního metabolomu

Do syntézy a metabolizmu steroidů je zapojená celá řada enzymů a poruchy jejich funkce mohou vést ke vzniku komplikací v těhotenství i mimo něj.

Neuroaktivní steroidy jsou významným faktorem v udržení stability fetoplacentární jednotky. Bylo prokázáno, že změny v koncentraci metabolitů úzce souvisí s časováním porodu [13]. Práce našeho týmu porovnávaly steroidní metabolom u žen, které porodily spontánně, se ženami, které rodily císařským řezem [14].

Důležitým proteohormonem, který se podílí na časování nástupu porodu a je v těhotenství produkován převážně placentou, je CRH. Jeho působení na fetoplacentární jednotku se ukazuje být jedním z klíčových kroků ovlivňujících trvání těhotenství a bývá některými autory označován jako tzv. placental clock. Je zajímavé, že jeho hladiny jsou nejvyšší těsně před nástupem porodu. Dle některých studií jsou hladiny CRH v době nástupu porodu až desettisíckrát vyšší v porovnání s hladinami na počátku těhotenství [15–17]. Těsně před porodem dochází ke konverzi „relaxačních" izoforem receptorů pro CRH za ty, které pozitivně ovlivňují kontraktilitu myomet-

ria. Je potvrzeno, že vysoké hladiny CRH v průběhu porodu ovlivňují funkci oxytocinu a prostaglandinu F2α, a tím dále působí na kontraktilitu myometria [18–20].

Procesem popsaným výše lze vysvětlit endokrinní načasování začátku porodu, vč. předčasného nástupu děložní činnosti.

Ve studii Hilla et al byly na základě hladin endogenních steroidů v cirkulaci matky, pupečníkové arteriální a venózní krvi a v plodové vodě navrženy modely pro predikci nástupu porodu, které ukázaly, že s nástupem porodu souvisí celá řada těchto látek. Korelace byly nejtěsnější pro pupečníkovou krev, avšak potenciálně nejlépe prakticky využitelný model založený na steroidomu v mateřské krvi byl stále velmi efektivní, zatímco korelace se steroidy v plodové vodě byly o něco slabší. Je zřejmé, že v porovnání s pupečníkovou krví či plodovou vodou je použití mateřské krve jednodušší a eticky přijatelnější [16].

Další intenzivně studovanou komplikací se vztahem ke steroidnímu metabolomu je intrahepatální cholestáza v těhotenství (ICP – intrahepatic cholestasis of pregnancy). V případech ICP je snížena produkce adrenokortikotropního hormonu (ACTH – adrenocorticotropic hormone) a kortizonu [21]. To poukazuje na poruchu osy hypotalamus – hypofýza. Naši autoři opakovaně popisují intenzivní vztah mezi touto osou a rozvojem ICP [22–24].

Studie vztahu neuroaktivních steroidů a krevního tlaku byly prováděny již v minulosti. Pregnanové steroidy se podílejí na regulaci systémového krevního tlaku. Někteří autoři prokázali vyšší sérové hladiny allopregnanolonu u žen s chronickou hypertenzí v porovnání s normotoničkami. V případech, kdy na hypertenzi nasedala preeklampsie, byly hladiny allopregnanolonu ještě vyšší. Výzkumy dále potvrdily, že hladiny progesteronu v plodové vodě byly vyšší u žen s pozdní formou preeklampsie [25,26].

Jednou z příčin vzniku preeklampsie je porucha regulace angiogenních a antiangiogenních faktorů v placentě. Estrogeny v těhotenství jsou převážně tvořeny konverzí sulfatovaných androgenů syntetizovaných ve fetální zóně fetální nadledviny. Porucha tvorby estrogenů vede k poruše angiogenních faktorů. Předpokládá se, že dysregulace tvorby estrogenů placentou přispívá k rozvoji preeklampsie [27].

Neuroaktivní steroidy rovněž působí na růst plodu *in utero*. Změny ve steroidním metabolomu mohou přispět k rozvoji fetální růstové restrikce [28]. Významný je vliv neuroaktivních steroidů na CNS plodu a matky.

Někteří autoři předpokládají, že v průběhu těhotenství se CNS plodu nachází ve stavu permanentního spánku a jeho aktivita je potlačena. Neuroaktivní steroidy v tomto případě působí jako neuroprotektivní faktory. Hladiny allopregnanolonu rostou při akutním hypoxickém stresu a chrání mozek plodu při porodu. Při chronické hypoxii se tyto mechanizmy neuplatňují, plod je tedy ohrožen více [29–31].

Řada studií prokázala, že nízké hladiny některých neuroaktivních steroidů přispívají ke vzniku depresí před porodem i *post partum* [32,33]. Souvislost mezi rozvojem depresí a hladinami allopregnanolonu v těhotenství a po porodu je ale patrná až ve 3. trimestru [34].

Neuroaktivní steroidy ovlivňují kromě CNS také periferní nociceptory. Působí jako blokátory kalciových kanálů a redukují vnímání bolesti [35]. Jde o jeden z mechanizmů přípravy těhotné na vnímání porodní bolesti.

Neuroaktivní steroidy lze využít i terapeuticky, např. u psychiatrických onemocnění – depresí, migrén, anxiózní poruchy osobnosti, panických atak a nespavosti.

Americká FDA (US Food and Drug Administration) v roce 2019 schválila použití brexanolonu (allopregnanolon) pro léčbu depresí a anxiózního syndromu [36]. Ten působí jako modulátor GABAA receptoru [37]. Terapie založená na neuroaktivních steroidech byla úspěšně využita i u léčby závislosti na nikotinu a kokainu, v běžné klinické praxi se ale nepoužívá [38].

V současnosti probíhá experimentální studie využití brexanolonu při léčbě akutní respirační tísně způsobené onemocněním covid-19 [39]. Na zvířecím modelu se dále zkoumá možnost využití ganaxolonu (derivát allopregnanolonu) při ovlivnění možného neurologického deficitu způsobeného předčasným porodem [40].

Stran komplexního účinku neuroaktivních steroidů je vhodné zmínit vzájemnou signalizaci s endokanabinoidním systémem. Progesteron a estrogen se prostřednictvím transkripčních faktorů podílí na tvorbě a degradaci enzymů udržujících hladinu **anandamidu**. Poruchy metabolizmu **anandamidu** úzce souvisí s poruchami implantace embrya, problematikou předčasného porodu a jsou v popředí experimentálního zájmu [41,42].

Vícečetné těhotenství

Vícečetné těhotenství je závažná porodnická komplikace. Četnost je v populaci vyjádřena tzv. Hellinovým pravidlem. Podle něj je nejčastější dvojčetné těhotenství s frekvencí v populaci 1 : 85 těhotenství. V rozvinutých zemích je četnost vyšší z důvodu častějšího využití metod asistované reprodukce.

Vícečetná těhotenství jsou ohrožena vysokým rizikem vzniku mnohých těhotenských patologií. Jednou z nejzávažnějších komplikací vícečetného těhotenství je předčasný porod [43,44].

Kromě toho jsou plody vícečetného těhotenství ohroženy:

- vyšší mírou růstové restrikce u alespoň jednoho z plodů,
- twin-to-twin transfuzním syndromem,
- respiratory distress syndromem (RDS),
- rizikem nitroděložního úmrtí plodu [45–47].

Endokrinologie plodů vícečetného těhotenství byla předmětem výzkumu několika studií. Cílem bylo popsat vliv pohlavních hormonů na jednotlivé plody.

Podkladem pro tyto studie byla obvykle tzv. twin testosteron transfer hypotéza. Ta tvrdí, že nadprodukce androgenů mužským plodem ovlivňuje negativně druhý plod a indukuje u něj maskulinní chování, vzhled a kognici. Tento efekt je častý u myšího modelu nebo u některých nižších savců [48].

Nizozemští autoři prokázali, že nedochází k ovlivnění ženských plodů ze smíšeného páru dvojčat testosteronem produkovaným matkou. Někteří autoři tvrdí, že androgeny matky jsou placentou plně konvertovány na estrogeny. Zdroj androgenů je v případě patologického ovlivnění plodu fetální [49]. Další studie tuto hypotézu nepotvrzují nebo přímo vyvrací [48,50].

Závěry jiných projektů opakovaně prokázaly, že dívky ze smíšeného páru dvojčat nejsou ovlivněny mužským sourozencem více v porovnání se situací, kdy jsou oba sourozenci dívky. Zjistilo se také, že zatímco těhotné ženy s dvojčaty měly vyšší hladiny estrogenů a progesteronu, u dětí byly hladiny v porovnání s jednočetným těhotenstvím nižší. Je to důkaz toho, že tyto děti nejsou exponovány vyšším hladinám pohlavních hormonů [51,52].

Dosavadní studie prováděné na plodech vícečetného těhotenství stanovovaly pouze omezený počet steroidních metabolitů, a to většinou pouze ze smíšené pupečníkové krve. Náš tým se v minulosti zabýval otázkou rozlišování mezi arteriální a venózní pupečníkovou krví [53]. Podrobné hodnocení steroidního metabolomu u vícečetného těhotenství zatím nebylo publikováno.

V současnosti na naší klinice probíhá studie hodnotící steroidní metabolom u dvojčat. Cílem je pokusit se:

a) podrobně popsat komponenty steroidního metabolomu u dvojčat,
b) nastínit rozdíl mezi vícečetným a jednočetným těhotenstvím,
c) určit, zda existují rozdíly v metabolizmu steroidů mezi dvojčaty různé chorionicity (tedy rozdíl v případě „společné" a „oddělené" placenty),
d) popsat rozdíl v hladinách v případě monozygotických (stejná DNA) a dizygotických (rozdílná DNA) bichoriálních dvojčat,
e) popsat mezipohlavní rozdíly v metabolomu, jelikož konverze prekurzorů u jednotlivých pohlaví je rozdílná,
f) popsat rozdíl v metabolomu plodů a matky v případě spontánního nástupu děložní činnosti a v případě porodu elektivním císařským řezem,
g) porovnat vztah k přidruženým patologiím, které jsme zatím sledovali pouze u jednočetného těhotenství (např. těhotenská cholestáza).

Péče o vícečetné těhotenství se řídí doporučeným postupem České gynekologicko-porodnické společnosti [54]. Náš výzkum je proto zajímavý i tím, že část předčasných porodů dvojčat je způsobena iatrogenně. Bude proto důležité pečlivě hodnotit jednotlivé případy a porovnávat je mezi sebou.

Závěr

Studium steroidního metabolomu ve vztahu ke komplikacím a patologiím v těhotenství je nezbytné k jejich pochopení a následnému možnému terapeutickému ovlivnění. Předpokládáme, že studiem steroidního metabolomu u dvojčat získáme přesnější pohled na vztah mezi plody a matkou, resp. mezi plody navzájem z hlediska steroidní endokrinologie a fyziologie i patofyziologie těhotenství.

Literatura

1. Strauss JF, Barbieri RB. Yen & Jaffe's reproductive endocrinology physiology, pathophysiology, and clinical management. 8th ed. Philadelphia: Elsevier 2019: 367.
2. Rupprecht R, Reul JM, Trapp T et al. Progesterone receptor-mediated effects of neuroactive steroids. Neuron 1993; 11(3): 523–530. doi: 10.1016/0896-6273(93)90156-I.
3. Tuem KB, Atey TM. Neuroactive steroids: receptor interactions and responses. Front Neurol 2017; 8: 442. doi: 10.3389/fneur.2017.00442.
4. Mitchell BF, Mitchell JM, Chowdhury J et al. Metabolites of progesterone and the pregnane X receptor: a novel pathway regulating uterine contractility in pregnancy? Am J Obstet Gynecol 2005; 192(4): 1304–1315. doi: 10.1016/j.ajog.2005.01.040.
5. Pasqualini JR, Chetrite GS. The formation and transformation of hormones in maternal, placental and fetal compartments: biological implications. Horm Mol Biol Clin Investig 2016; 27(1): 11–28. doi: 10.1515/hmbci-2016-0036.
6. Hill M, Parizek A, Cibula D et al. Steroid metabolome in fetal and maternal body fluids in human late pregnancy. J Steroid Biochem Mol Biol 2010; 122(4): 114–132. doi: 10.1016/j.jsbmb.2010.05.007.
7. Krone N, Hughes BA, Lavery GG et al. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). J Steroid Biochem Mol Biol 2010; 121(3–5): 496–504. doi: 10.1016/j.jsbmb.2010.04.010.
8. Haneef J, Shaharyar M, Husain A et al. Application of LC–MS/MS for quantitative analysis of glucocorticoids and stimulants in biological fluids. J Pharm Anal 2013; 3(5): 341–348. doi: 10.1016/j.jpha.2013.03.005.
9. Marcos J, Renau N, Casals G et al. Investigation of endogenous corticosteroids profiles in human urine based on liquid chromatography tandem mass spectrometry. Anal Chim Acta 2014; 812: 92–104. doi: 10.1016/j.aca.2013.12.030.
10. Dušková M, Kolátorová L, Šimková M et al. Steroid diagnostics of 21st century in the light of their new roles and analytical tools. Physiol Res 2020; 69(Suppl 2): S193–S203. doi: 10.33549/physiolres.934517.
11. Yuan TF, Le J, Wang ST et al. An LC/MS/MS method for analyzing the steroid metabolome with high accuracy and from small serum samples. J Lipid Res 2020; 61(4): 580–586. doi: 10.1194/jlr.D119000591.
12. Hill M, Hána V Jr, Velíková M et al. A method for determination of one hundred endogenous steroids in human serum by gas chromatography – tandem mass spectrometry. Physiol Res 2019; 68(2): 179–207. doi: 10.33549/physiolres.934124.
13. Pašková A. Úloha neuroaktivních steroidů v těhotenství a jejich význam v prenatální diagnostice. Disertační práce. Gynekologicko-porodnická klinika 1. LF UK a VFN v Praze 2013.
14. Adamcová K, Kolátorová L, Škodová T et al. Steroid hormone levels in the peripartum period – differences caused by fetal sex and delivery type. Physiol Res 2018; 67(Suppl 3): S489–S497. doi: 10.33549/physiolres.934019.
15. Ellis MJ, Livesey JH, Inder WJ et al. Plasma corticotropin-releasing hormone and unconjugated estriol in human pregnancy: gestational patterns and ability to predict preterm delivery. Am J Obstet Gynecol 2002; 186(1): 94–99. doi: 10.1067/mob.2002.119188.
16. Hill M, Parizek A, Kancheva R et al. Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and

in amniotic fluid in normal and preterm labor. J Steroid Biochem Mol Biol 2010; 121(3–5): 594–610. doi: 10.1016/j.jsbmb.2009.10.012.
17. McLean M, Smith R. Corticotrophin-releasing hormone and human parturition. Reproduction 2001; 121(4): 493–501. doi: 10.1530/rep.0.1210493.
18. Ravanos K, Dagklis T, Petousis S et al. Factors implicated in the initiation of human parturition in term and preterm labor: a review. Gynecol Endocrinol 2015; 31(9): 679–683. doi: 10.3109/09513590.2015.1076783.
19. Linton EA, Woodman JR, Asboth G et al. Corticotrophin releasing hormone: its potential for a role in human myometrium. Exp Physiol 2001; 86(2): 273–281. doi: 10.1113/eph8602183.
20. Grammatopoulos D, Thompson S, Hillhouse EW. The human myometrium expresses multiple isoforms of the corticotropin-releasing hormone receptor. J Clin Endocrinol Metab 1995; 80(8): 2388–2393. doi: 10.1210/jcem.80.8.7629235.
21. Zhu P, Tao FB, Jiang XM et al. Effect of intrahepatic cholestasis of pregnancy on the functions of hypothalamic-pituitary-adrenocortical axis and adrenal cortex in normal neonates. Zhongguo Dang Dai Er Ke Za Zhi 2010; 12(1): 5–8.
22. Pařízek A, Dušková M, Vítek L et al. The role of steroid hormones in the development of intrahepatic cholestasis of pregnancy. Physiol Res 2015; 64(Suppl 2): S203–S209. doi: 10.33549/physiolres.933117.
23. Pařízek A, Hill M, Dušková M et al. A comprehensive evaluation of steroid metabolism in women with intrahepatic cholestasis of pregnancy. PLoS One 2016; 11(8): e0159203. doi: 10.1371/journal.pone.0159203.
24. Šimják P, Pařízek A, Vítek L et al. Fetal complications due to intrahepatic cholestasis of pregnancy. J Perinat Med 2015; 43(2): 133–139. doi: 10.1515/jpm-2014-0089.
25. Parker CR Jr, Everett RB, Quirk JG Jr et al. Hormone production during pregnancy in the primigravid patient. I. Plasma levels of progesterone and 5-alpha-pregnane-3,20-dione throughout pregnancy of normal women and women who developed pregnancy-induced hypertension. Am J Obstet Gynecol 1979; 135(6): 778–782.
26. Luisi S, Petraglia F, Benedetto C et al. Serum allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patients. J Clin Endocrinol Metab 2000; 85(7): 2429–2433. doi: 10.1210/jcem.85.7.6675.
27. Berkane N, Liere P, Oudinet JP et al. From pregnancy to preeclampsia: a key role for estrogens. Endocr Rev 2017; 38(2): 123–144. doi: 10.1210/er.2016-1065.
28. Baud O, Berkane N. Hormonal changes associated with intra-uterine growth restriction: impact on the developing brain and future neurodevelopment. Front Endocrinol (Lausanne) 2019; 10: 179. doi: 10.3389/fendo.2019.00179.
29. Hirst JJ, Kelleher MA, Walker DW et al. Neuroactive steroids in pregnancy: key regulatory and protective roles in the foetal brain. J Steroid Biochem Mol Biol 2014; 139: 144–153. doi: 10.1016/j.jsbmb.2013.04.002.
30. Hirst JJ, Palliser HK, Yates DM et al. Neurosteroids in the fetus and neonate: potential protective role in compromised pregnancies. Neurochem Int 2008; 52(4–5): 602–610. doi: 10.1016/j.neuint.2007.07.018.
31. Hirst JJ, Yawno T, Nguyen P et al. Stress in pregnancy activates neurosteroid production in the fetal brain. Neuroendocrinology 2006; 84(4): 264–274. doi: 10.1159/000097990.
32. Deligiannidis KM, Kroll-Desrosiers AR, Mo S et al. Peripartum neuroactive steroid and γ-aminobutyric acid profiles in women at-risk for postpartum depression. Psychoneuroendocrinology 2016; 70: 98–107. doi: 10.1016/j.psyneuen.2016.05.010.
33. McEvoy K, Payne JL, Osborne LM. Neuroactive steroids and perinatal depression: a review of recent literature. Curr Psychiatry Rep 2018; 20(9): 78. doi: 10.1007/s11920-018-0937-4.
34. Hellgren C, Comasco E, Skalkidou A et al. Allopregnanolone levels and depressive symptoms during pregnancy in relation to single nucleotide polymorphisms in the allopregnanolone synthesis pathway. Horm Behav 2017; 94: 106–113. doi: 10.1016/j.yhbeh.2017.06.008.
35. Todorovic SM, Pathirathna S, Brimelow BC et al. 5beta-reduced neuroactive steroids are novel voltage-dependent blockers of T-type Ca^{2+} channels in rat sensory neurons in vitro and potent peripheral analgesics in vivo. Mol Pharmacol 2004; 66(5): 1223–1235. doi: 10.1124/mol.104.002402.
36. Zorumski CF, Paul SM, Covey DF et al. Neurosteroids as novel antidepressants and anxiolytics: GABA-A receptors and beyond. Neurobiol Stress 2019; 11: 100196. doi: 10.1016/j.ynstr.2019.100196.
37. Belelli D, Hogenkamp D, Gee KW et al. Realising the therapeutic potential of neuroactive steroid modulators of the GABAA receptor. Neurobiol Stress 2019; 12: 100207. doi: 10.1016/j.ynstr.2019.100207.
38. Anker JJ, Carroll ME. The role of progestins in the behavioral effects of cocaine and other drugs of abuse: human and animal research. Neurosci Biobehav Rev 2010; 35(2): 315–333. doi: 10.1016/j.neubiorev.2010.04.003.
39. Good clinical practice network. Studie brexanolonu pro akutní syndrom respirační tísně v důsledku covid-19. 2021 [online]. Available from: https://ichgcp.net/cs/clinical-trials-registry/NCT04 537806.
40. Shaw JC, Dyson RM, Palliser HK et al. Neurosteroid replacement therapy using the allopregnanolone-analogue ganaxolone following preterm birth in male guinea pigs. Pediatr Res 2019; 85(1): 86–96. doi: 10.1038/s41390-018-0185-7.
41. Karasu T, Marczylo TH, Maccarrone M et al. The role of sex steroid hormones, cytokines and the endocannabinoid system in female fertility. Hum Reprod Update 2011; 17(3): 347–361. doi: 10.1093/humupd/dmq058.
42. Santoro A, Mele E, Marino M et al. The complex interplay between endocannabinoid system and the estrogen system in central nervous system and periphery. Int J Mol Sci 2021; 22(2): 972. doi: 10.3390/ijms22020972.
43. Chauhan SP, Scardo JA, Hayes E et al. Twins: prevalence, problems, and preterm births. Am J Obstet Gynecol 2010; 203(4): 305–315. doi: 10.1016/j.ajog.2010.04.031.
44. Sentilhes L, Oppenheimer A, Bouhours AC et al. Neonatal outcome of very preterm twins: policy of planned vaginal or cesarean delivery. Am J Obstet Gynecol 2015; 213(1): 73.e1–73.e7. doi: 10.1016/j.ajog.2015.02.020.
45. Townsend R, Khalil A. Fetal growth restriction in twins. Best Pract Res Clin Obstet Gynaecol 2018; 49: 79–88. doi: 10.1016/j.bpobgyn.2018.02.004.
46. Bamberg C, Hecher K. Update on twin-to-twin transfusion syndrome. Best Pract Res Clin Obstet Gynaecol 2019; 58: 55–65. doi: 10.1016/j.bpobgyn.2018.12.011.
47. Canpolat FE, Yurdakök M, Korkmaz A et al. Birthweight discordance in twins and the risk of being heavier for respiratory distress syndrome. Twin Res Hum Genet 2006; 9(5): 659–663. doi: 10.1375/183242706778553372.

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48. Tapp AL, Maybery MT, Whitehouse AJ. Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. Horm Behav 2011; 60(5): 713–722. doi: 10.1016/j.yhbeh.2011.08.011.
49. Cohen-Bendahan CC, van Goozen SH, Buitelaar JK et al. Maternal serum steroid levels are unrelated to fetal sex: a study in twin pregnancies. Twin Res Hum Genet 2005; 8(2): 173–177. doi: 10.1375/1832427053738764.
50. Talia C, Raja EA, Bhattacharya S et al. Testing the twin testosterone transfer hypothesis-intergenerational analysis of 317 dizygotic twins born in Aberdeen, Scotland. Hum Reprod 2020; 35(7): 1702–1710. doi: 10.1093/humrep/deaa091.
51. Kuijper EA, Twisk JW, Korsen T et al. Mid-pregnancy, perinatal, and neonatal reproductive endocrinology: a prospective cohort study in twins and singleton control subjects. Fertil Steril 2015; 104(6): 1527–1534. doi: 10.1016/j.fertnstert.2015.08.016.
52. Thomas HV, Murphy MF, Key TJ et al. Pregnancy and menstrual hormone levels in mothers of twins compared to mothers of singletons. Ann Hum Biol 1998; 25(1): 69–75. doi: 10.1080/03014469800005432.
53. Pašková A, Pařízek A, Hill M et al. Steroid metabolome in the umbilical cord: is it necessary to differentiate between arterial and venous blood? Physiol Res 2014; 63(1): 115–126. doi: 10.33549/physiolres.932624.
54. Doporučený postup České gynekologicko-porodnické společnosti. Vedení porodu vícečetného těhotenství. 2013 [online]. Dostupné z: https://www.lekaridnes.cz/wp-content/uploads/2016/08/p-2013-vedeni-porodu-vicecetneho-tehotenstvi.pdf.

ORCID autorů
A. Černý 0000-0001-9230-9184
Z. Laštůvka
M. Hill
A. Pařízek

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MUDr. Andrej Černý
Gynekologicko-porodnická klinika
1. LF UK a VFN v Praze
Apolinářská 18
128 08 Praha 2
andrej.cerny@gmail.com

A Method for Determination of One Hundred Endogenous Steroids in Human Serum by Gas Chromatography-Tandem Mass Spectrometry

M. HILL[1], V. HÁNA Jr.[2], M. VELÍKOVÁ[1], A. PAŘÍZEK[3], L. KOLÁTOROVÁ[1], J. VÍTKŮ[1], T. ŠKODOVÁ[1], M. ŠIMKOVÁ[1], P. ŠIMJÁK[3], R. KANCHEVA[1], M. KOUCKÝ[3], Z. KOKRDOVÁ[3], K. ADAMCOVÁ[3], A. ČERNÝ[3], Z. HÁJEK[3], M. DUŠKOVÁ[1], J. BULANT[1,4,5], L. STÁRKA[1]

[1]Department of Steroid Hormones and Proteohormones, Institute of Endocrinology, Prague, Czech Republic, [2]Third Internal Clinic – Clinic of Endocrinology and Metabolism, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic, [3]Department of Gynecology and Obstetrics, General University Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic, [4]Department of Psychiatry, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic, [5]Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

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Summary

Steroid profiling helps various pathologies to be rapidly diagnosed. Results from analyses investigating steroidogenic pathways may be used as a tool for uncovering pathology causations and proposals of new therapeutic approaches. The purpose of this study was to address still underutilized application of the advanced GC-MS/MS platform for the multicomponent quantification of endogenous steroids. We developed and validated a GC-MS/MS method for the quantification of 58 unconjugated steroids and 42 polar conjugates of steroids (after hydrolysis) in human blood. The present method was validated not only for blood of men and non-pregnant women but also for blood of pregnant women and for mixed umbilical cord blood. The spectrum of analytes includes common hormones operating *via* nuclear receptors as well as other bioactive substances like immunomodulatory and neuroactive steroids. Our present results are comparable with those from our previously published GC-MS method as well as the results of others. The present method was extended for corticoids and 17α-hydroxylated 5α/β-reduced pregnanes, which are useful for the investigation of alternative "backdoor" pathway. When comparing the analytical characteristics of the present and previous method, the first exhibit by far higher selectivity, and generally higher sensitivity and better precision particularly for 17α-hydroxysteroids.

Key words

Steroid metabolome • Human blood • Gas chromatography-tandem mass spectrometry • Backdoor pathway • Pregnancy • Mixed umbilical cord blood

Corresponding author

M. Hill, Department of Steroid Hormones and Proteohormones, Institute of Endocrinology, Národní 8, 116 94, Prague, Czech Republic. E-mail: mhill@endo.cz

Introduction

For almost six decades, gas chromatography-mass spectrometry (GC-MS) served as an efficient tool for the routine quantification of endogenous steroids (Hill *et al.* 2010a, Hill *et al.* 2010b, Krone *et al.* 2010). At present, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is also widely used and has become the gold standard for steroid quantification (Soldin and Soldin 2009). A number of LC-MS/MS based steroidomics studies was primarily focused on

Fax +420 241 062 164, e-mail: physres@fgu.cas.cz, www.biomed.cas.cz/physiolres

corticosteroids and their metabolites (Gomes *et al.* 2009, Haneef *et al.* 2013, Marcos *et al.* 2014). Other chromatographic strategies may involve a direct LC-MS/MS detection of unaltered glucuronoconjugated metabolites (Esquivel *et al.* 2017) or the use of supercritical fluids for extraction of steroidome (Kureckova *et al.* 2002). However, in steroid metabolomics (steroidomics), GC-MS remains the method of choice (Krone *et al.* 2010). A more advanced and therefore more sensitive, specific and precise GC-MS platform known as gas-chromatography tandem-mass spectrometry (GC-MS/MS) has lately been developed. The GC-MS/MS platform on the one hand retains the advantages of GC-MS in precisely distinguishing isomers with the same mass to charge ratio (m/z). However, the use of GC-MS/MS in the analysis of endogenous steroids has still been limited. Current studies using the GC-MS/MS platform have mostly focused on the quantification of anabolic steroids in the blood of athletes or farmyard animals (Gambelunghe *et al.* 2007, Impens *et al.* 2007, Marcos *et al.* 2002, Raro *et al.* 2016, Rossi *et al.* 1994, Shen *et al.* 2008, Van Vyncht *et al.* 1994, Wong *et al.* 2017, Yamada *et al.* 2008) or on steroid quantifications in wastewaters (Andrasi *et al.* 2013, Kelly 2000, Trinh *et al.* 2011, Zuehlke *et al.* 2005). Blokland *et al.* (2012) simultaneously quantified 47 steroids in the form of unconjugated steroids, glucuronides and sulfates in bovine urine. Regarding the number of steroids detected, the lead is still held by a series of studies from Christakoudi and coworkers who identified and quantified human urinary steroids. Their first study included 146 C21 steroids (Christakoudi *et al.* 2010), the second one 32 additional C21 steroids (Christakoudi *et al.* 2012a), the third 76 C19 steroids (Christakoudi *et al.* 2012b) and the fourth study additional 52 C21 steroids (Christakoudi *et al.* 2013). These studies have provided a complex qualitative picture of the urinary steroid metabolome in humans; however, the lack of validation of the methods used remains its weakness. The authors from research group headed by Man-Ho Choi (Molecular Recognition Research Center of Korea Institute of Science and Technology) published a series of extensive metabolomic studies on the GC-MS platform, which were focused on the role of urinary steroids in human physiology and pathophysiology (Ha *et al.* 2009, Choi and Chung 2014, Kim *et al.* 2013, Moon *et al.* 2016, Moon *et al.* 2009). There are few GC-MS/MS studies focused on circulating steroids in humans and other mammals, and all have quantified a limited number of steroids (Courant *et al.* 2010, Hansen *et al.* 2011, Matysik and Schmitz 2015, Nilsson *et al.* 2015, Styrishave *et al.* 2017).

The purpose of this study was to address the application of the GC-MS/MS platform for the simultaneous quantification of endogenous steroids. We developed and validated a GC-MS/MS method for the multicomponent quantification of unconjugated steroids and their polar conjugates (after hydrolysis). Of the original 120 steroids or their polar conjugates tested, only 100 of them met validation criteria for at least some physiological situations. Our method was validated not only for blood of men and non-pregnant women but also for blood of pregnant women and for umbilical cord blood. The spectrum of analytes in our method includes precursor steroids, active steroids and steroid metabolites, and covers the vast part of steroid metabolome in humans (Figs 1 and 2). Steroid profiling helps various pathologies to be rapidly diagnosed. Moreover, the results from analyses investigating steroidogenic pathways may be used as a tool for uncovering pathology causations and proposals of new therapeutic approaches (Bicikova *et al.* 2013, Hill *et al.* 2010c, Kanceva *et al.* 2015, Parizek *et al.* 2016, Sosvorova *et al.* 2015, Sterzl *et al.* 2017, Vankova *et al.* 2016).

Methods

Samples

Serum samples from non-pregnant subjects were collected from the employees of the Institute of Endocrinology, Prague, Czech Republic and their relatives, as well as from patients of the Institute of Endocrinology. Serum samples from pregnant women and umbilical cord serum at birth were obtained from patients of the Department of Gynecology and Obstetrics, General University Hospital and 1st Faculty of Medicine of Charles University in Prague. For all participants, the clinical protocol was approved by the Ethics Committee of the Institute of Endocrinology and by the Ethics Committee of the General University Hospital and 1st Faculty of Medicine of Charles University in Prague. Informed written consent was obtained from all participants. Serum from blood was obtained after centrifugation (5 min at 2,000 × g at 2 °C), and stored at -20 °C until analyzed.

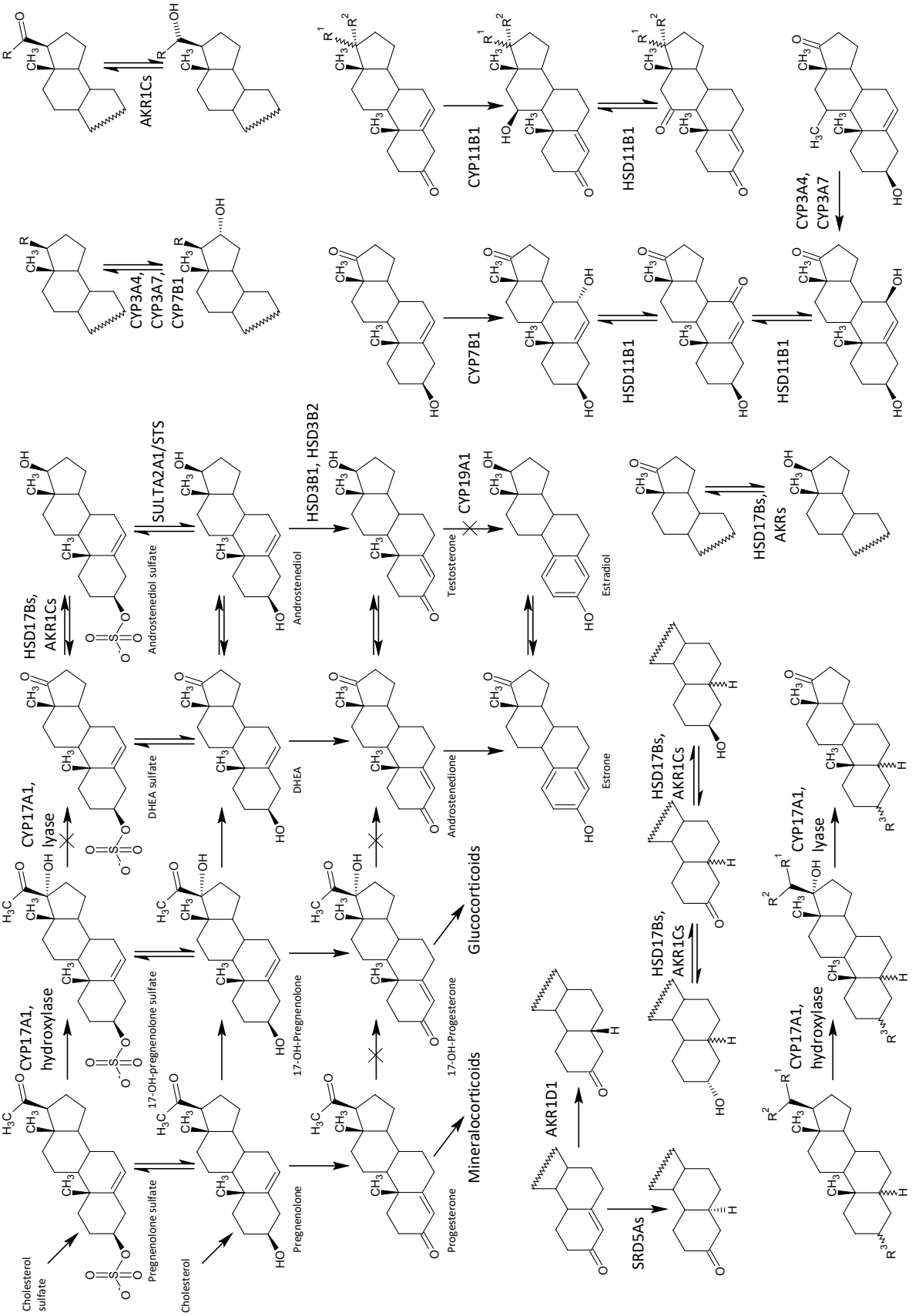

Fig. 1. Simplified scheme of human steroidogenesis. The symbol x signifies the minor or absent metabolic pathway in humans.

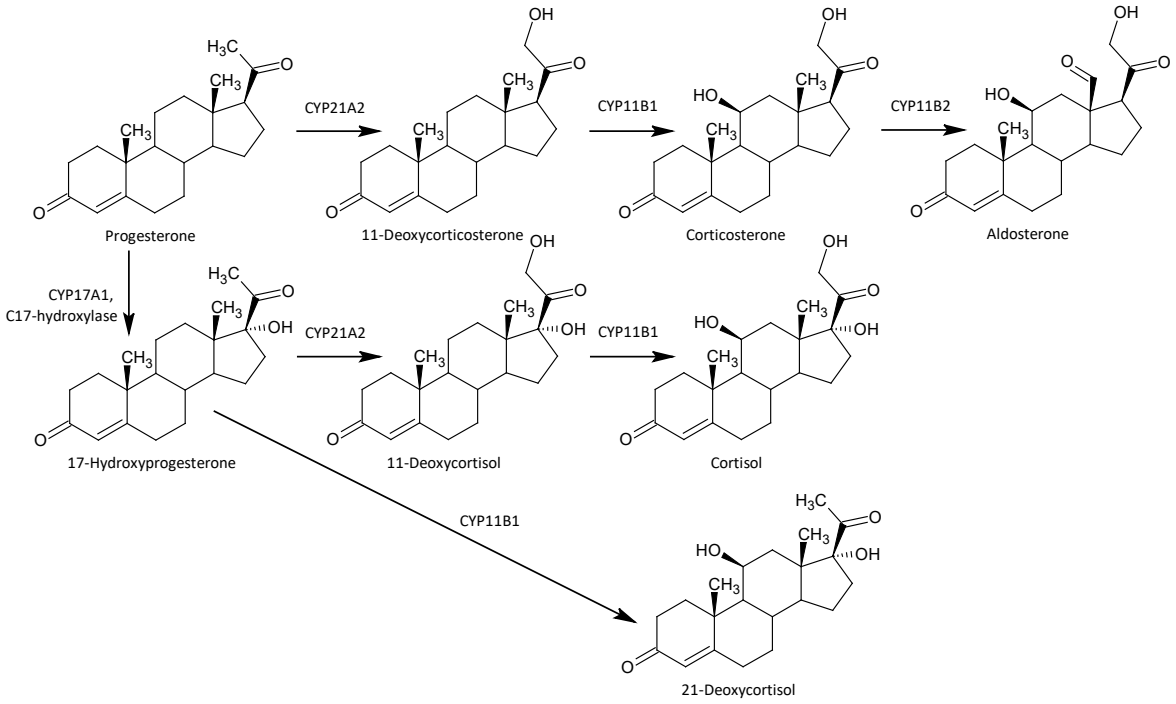

Fig. 2. Simplified scheme of corticosteroid pathways in human.

Chemicals

Most steroids and deuterated standards were purchased from Steraloids (Newport, RI, USA). The deuterated standard D7 cortisone [2,2,4,6,6,12,12-D7] and trimethylchlorosilane (TMCS) for hydrolysis of steroids conjugates were from Sigma-Aldrich (St. Louis, USA). Sylon BTZ, methoxyamine hydrochloride and all other solvents and chemicals were from Merck (Darmstadt, Germany). All solvents were of HPLC grade.

Stock solutions, calibration standards, and quality control samples

Stock solutions of external and internal standards (ISs) were prepared in methanol at the concentration of 1 mg/ml. The calibration curve samples (charcoal-stripped plasma with internal and external standards) were prepared in triplicate, blank samples (charcoal-stripped plasma without ISs) were made separately for unconjugated and conjugated steroids as well as zero samples (charcoal-stripped serum with ISs) were prepared. Charcoal-stripped serum was made using a multistep adsorption of steroids on charcoal. The absence of steroids in this matrix was checked by spiking of serum with [^{3}H]cortisol (10,000 dpm/ml) and measurement of the residual radioactivity close to zero. In brief, 100 g of Activated Charcoal Norit from Sigma-Aldrich (St. Louis, USA) was mixed with 1 liter of deionized water and let overnight. Then the water with fine particles of the charcoal was decanted, the charcoal was spread out on the filtration paper and let overnight. Then the charcoal was dried at 200 °C in glass baking bowl for 2 h. The dried charcoal was stored in wide mouth glass reagent bottle. Afterwards, 10^7 dpm of 3H cortisol from NEN® Life Science Products (Boston, MA, USA) was added to 1 liter of pooled human serum and 200 µl of the mixture was measured in triplicate in scintillation counter (1,000-2,000 dpm). Than the charcoal (50 g) was mixed with the pooled serum at 4 °C for 3 h. Then the centrifugation in cooled centrifuge followed at 4 °C for 20 min (3,500 rpm). Subsequently, the supernatant was decanted and filtered across the folded filter paper in refrigerator and the filtrate is then mixed with further 50 g of the charcoal overnight in the refrigerator and afterwards the further filtration followed. The filtrate was then treated (in parts) at 84,000 g in ultracentrifuge at 4 °C for 25 min and the centrifugation was repeated until the serum was free of charcoal particles. Finally, the 200 µl of the treated serum was measured (in triplicate) for 3H radioactivity together with the 200 µl of water (in triplicate) as negative control and the results were compared with initial activity of the 3H cortisol spiked serum.

Quality control (QC) samples were prepared using different serum pools from adult men, women in follicular menstrual phase and women in luteal menstrual phase, pregnant women (week 28-42 of pregnancy) and

from mixed umbilical cord serum, which was collected at labor (week 28-42 of pregnancy). Using five pools differing according to gender, menstrual phase, pregnancy status and matrix (mixed umbilical serum) the QC control samples contained substantially different steroid levels covering gender differences and distinct physiological status in women. The number of samples in mixed pools in individual groups out of pregnancy was greater than 100 for each group, while the sample numbers for the groups of pregnant women and mixed umbilical serum were greater than 30 for each group.

From each stock solution of steroid (1 mg/ml), 10 µl was added into the glass tube. The mixture was dried in vacuum centrifuge (2 h). Then the stock solutions for calibration samples were prepared in concentrations 5,000, 1,000, 250, 62.5, 15.625, 3.906, 0.977, 0.244, 0.061 ng/ml in methanol. From these stock solutions 100 µl was administered to 10 ml extraction glass tubes vials and the mixtures were dried in the vacuum centrifuge at 45 °C. Then 1 ml of charcoal-stripped serum and the solutions were mixed for 1 min. The next steps were identical for the calibration samples, zero samples, quality control samples and serum samples. The amount of 15 µl from the mixed stock solution containing ISs was added to the aforementioned samples. The mixed stock solution of ISs for quantification of unconjugated steroids was prepared from the stock solutions of individual ISs as follows: 10 µl D6-dehydroepiandrosterone (D6-DHEA) ([2,2,3,4,4,6-D6]-DHEA, 1 mg/ml), 10 µl D8-Prog17 ([2,2,4,6,6,21,21,21-D8]-17α-hydroxyprogesterone, 1 mg/ml), 10 µl D9-Prog ([2,2,4,6,6,17α,21,21,21-D9]-progesterone, 1 mg/ml), 100 µl D4-cortisol ([9,11,12, 12-D4)-cortisol, 1 mg/ml), 50 µl D7-cortisone ([2,2,4,6,6, 12,12-D7]-cortisone, 10 µg/ml) were mixed, the mixture was dried under the flow of nitrogen and the dry residue was dissolved in 1 ml of methanol. The internal standard of D6-DHEA sulfate ([2,2,3,4,4,6-D6]-DHEA sulfate, 1 mg/ml) for quantification of conjugated steroids was prepared similarly. The volume of 50 µl D6-DHEA sulfate, 1 mg/ml) was dried under the flow of nitrogen and the dry residue was dissolved in 1 ml of methanol.

Sample preparation

The sample preparation proceeded as follows: after addition of 15 µl of the mixed stock solution of ISs for quantification of unconjugated steroids to 1 ml of serum fluid and mixing (1 min), the unconjugated steroids were extracted from 1 ml of the mixture with diethyl-ether (3 ml). The diethyl-ether extract was dried in a block heater at 37 °C. The lipids in the dry residue of the diethyl-ether extract were separated by partitioning between a mixture of methanol with water 4:1 (1 ml) and pentane (1 ml). The pentane phase was discarded and the polar phase was dried in a vacuum centrifuge at 60 °C (2 h). The dry residue from the polar phase was firstly dissolved in 100 µl of acetonitrile. The solution was transferred into the 1 ml conical vial and dried in the flow of nitrogen. The dry residue was derivatized first with a methoxyamine hydrochloride solution in pyridine (2 %) (60 °C, 1 h) to convert the oxo-groups to methyloxime derivatives. After this first derivatization, the mixture was dried in a flow of nitrogen and the dry residue was treated with the reagent Sylon BTZ (90 °C, 24 h). The Sylon BTZ is a mixture of N,O-bis(trimethylsilyl)acetamide (BSA) + trimethylchlorosilane (TMCS) + N-trimethyl-silylimidazole (TMSI) (3:2:3). This sylilating agent forms trimethylsilyl derivatives on hydroxy-groups (TMS-MOX derivatives). After this second derivatization step, the mixture was dried in the nitrogen flow (2 min). After administration of approximately 1 mg of ammonium bicarbonate, the residue was partitioned between isooctane (100 µl) and N,N-dimethylformamide (50 µl). Then the volume of the vial was mixed (1 min) and centrifuged for 20 min at 3,000 rpm. The lower, polar layer was aspirated with a Pasteur pipette and the upper non-polar layer remained in the vial for GC-MS/MS analysis. From the upper layer, 2 µl was injected into the GC-MS/MS system.

Steroid conjugates remaining in the polar residue after diethyl ether extractions were analyzed as follows: The volume of 15 µl D6-DHEA sulfate solution (50 µg/ml) was mixed with this residue (1 min mixing). Then 1 ml of methanol was added and mixed for additional 1 min. After the centrifugation of the mixture (20 min at 3,000 rpm), the upper layer was transferred to the clean 10 ml extraction tube, dried in the vacuum centrifuge at 37 °C (5 h), and the dry residues were chemically hydrolyzed according to Dehennin and Peres (1996). Briefly, 1 ml of 1 M TMCS was added to the dry residue of the upper layer and after 1 min mixing, the hydrolysis proceeded for 1 h at 55 °C. Then 100 mg of sodium bicarbonate was added and after short mixing, the hydrolyzed samples were again dried in the vacuum centrifuge at 37 °C (5 h). The dried residues were reconstituted with 500 µl of chromatographic water and then further processed in the same way as the free steroids. The calibration samples for the conjugated steroids were prepared similarly as for their unconjugated

analogues but the standards were mixed with the polar residues after diethyl ether extraction instead of the 1 ml of charcoal-stripped serum.

Instruments and chromatography conditions

Instrument settings

The instrument used was a GCMS-TQ8040 system from Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with an automatic flow control, an AOC-20s autosampler and a triple quadrupole detector with an adjustable electron voltage of 10-195 V. The analysis was conducted in multiple reaction monitoring (MRM) mode. A capillary column with a medium polarity RESTEK Rtx-50 column (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron-impact ionization with electron voltage fixed at 60 V and emission current set to 151 μA was used for the measurements. The temperatures of the injection port, ion source and interface were maintained at 220, 300, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set to 3 ml/min. The samples were injected using a high-pressure mode, which was applied at 200 kPa and maintained for 1 min. The detector voltage was set to 2.2 kV. The temperature program was as follows: 1 min delay at 80 °C, increase to 190 °C (40 °C/min), increase to 210 °C (6 °C/min), increase to 300 °C (20 °C/min), increase to 320 °C (40 °C/min), 4 min delay at 320 °C, initial pressure 34 kPa, injector temperature 220 °C, analysis duration 16.08 min.

Optimization of method sensitivity

To optimize method sensitivity, the analysis was carried out using two separately injected aliquots (2 μl) for two different groups of steroids for each sample (Table 1). The injection volume of samples was 2 μl. However, two steroid sulfates injected in the second aliquot exceeded the upper limit of linear dynamic range (LDR). To quantify these analytes, this measurement was repeated using the third aliquot with reduced injection volume (0.2 μl). The list of analytes with corresponding abbreviations, correlation coefficients (characterizing the linearity of the response) and the respective LDRs with indication of the abundant steroid conjugates quantified in the third aliquot are shown in Table 2.

For further improvement of sensitivity, the method used time-programmed MRM acquisition. The number of injection aliquot, number of time-programmed MRM acquisition window (AW), MRM transitions with corresponding optimum collision energies for individual steroids and ISs for the corresponding steroids are shown in Table 1. The optimization of collision energies for individual steroids was performed using the Microsoft Excel Macro-Enabled Worksheet named "MRM Optimization Tool" from Shimadzu (Kyoto, Japan).

The number of qualifiers ranged from no qualifier to three qualifiers with respect to the fragmentation patterns of individual steroid derivatives and sensitivity of the method, which is inversely related with the number of MRM transitions in the given AW (Table 1). For instance, in the case of 21-deoxycortisol (DOF) just a single MRM transition was selected 517>427 (12 V) as the quantifier without a qualifier, because only this transition had a satisfactory response (Table 1). The case of PD3β5α20α was similar. In addition, the respective AW 7 included a relatively high number of transitions, which limited the sensitivity. On the other hand, in the AW 1, the androstanediols were measured using three confirmation MRM transitions as the total number of transitions in AW 1 was low (Table 1).

Selection of internal standards

To represent different chemical and physical properties of various steroid molecules we originally tried to use a maximum number of available ISs. However, we also respected the number of deuterium atoms in the steroid molecule, which is sufficient for separation of the signals from non-deuterated steroid and its deuterated counterpart and, at the same time, wide concentration range of steroids in serum samples, and isotopic purity of the ISs. In addition, we also considered an inverse relationship between the number of MRM-transitions in acquisition windows and sensitivity of the assay. Therefore, from the original number of 16 deuterated steroids we selected five deuterated standards with different polarity such as D6-DHEA sulfate (IS1), D6-DHEA (IS2), D8-Prog17 (IS3), D9-Prog (IS4), D4-cortisol (IS5), and D7-cortisone (IS6). For the conjugated steroids, only IS1 was applicable, because the remaining ISs were instable during the hydrolysis. Therefore, for the quantification of steroid conjugates, the IS1 was used instead of IS3 and IS4 (Table 1).

Table 1. MRM acquisition windows (MRM-AW), retention times, transitions and optimum collision energies for individual steroids.

Injection	MRM-AW	Steroid	IS[a]	Retention time [min]				MRM transition (collision energy [V])			
				peak 1	peak 2	peak 3	peak 4	MRM transition 1	MRM transition 2	MRM transition 3	MRM transition 4
1	1	5β-Pregnane-3α,17α,20α-triol	3(1[a])	8.34				435>255 (12)	345>255 (9)		
1	1	5α-Pregnane-3α,17α,20α-triol	3(1[a])	8.37				435>255 (12)	345>255 (9)		
1	1	17α-Hydroxypregnanolone	3(1[a])	8.48				492>172 (24)	476>386 (12)	476>296 (15)	
1	1	17α-Hydroxyallopregnanolone	3(1[a])	8.59				492>172 (24)	476>386 (12)	476>296 (15)	
1	1	D6-DHEA sulfate (IS1, conjugates)		8.61				364>274 (9)			
1	1	D6-DHEA (IS2)		8.61				364>274 (9)			
1	1	11β-Hydroxyandrosterone	1	8.65				448>268 (12)	448>358 (9)		
1	1	11β-Hydroxyetiocholanolone	1	8.70				448>268 (12)	448>358 (9)		
1	2	5α-Pregnane-3β,17α,20α-triol	3(1[a])	9.00				435>255 (12)	345>255 (9)		
1	3	11β-Hydroxyepiandrosterone	1	9.19				448>268 (12)	448>147 (18)		
1	3	Estrone	1	9.37				371>340 (9)	340>231 (15)		
1	4	3α,5α-Tetrahydrocorticosterone	1	9.56				564>158 (18)	474>158 (18)		
1	4	3α,5β-Tetrahydrocorticosterone	1	9.60				564>158 (18)	474>158 (18)		
1	5	17α,20α-Dihydroxy-4-pregnen-3-one	3(1[a])	10.00	10.06			388>298 (9)	388>267 (12)	298>145 (15)	
1	6	21-Deoxycortisol	1	10.03	10.36			517>427 (12)			
1	6	D8-17α-Hydroxyprogesterone (IS3)		10.14	10.21			437>377 (18)			
1	6	11β-Hydroxyandrostenedione	2	10.20	10.32			401>279 (9)	401>311 (9)		
1	6	D9-Progesterone (IS4)		10.41	10.49			381>350 (9)			
1	7	D4-Cortisol (IS5)		10.69	10.76			609>519 (15)			
1	7	Cortisol	5	10.70	10.78			605>515 (12)	605>143 (21)	515>425 (15)	
1	7	11-Deoxycorticorticosterone	4(1[a])	10.76	10.84			460>286 (12)	429>298 (9)		
1	8	D7-Cortisone (IS6)		10.93	10.96			538>168 (18)			
1	8	Corticosterone	1	10.94	11.03	11.13	11.22	427>293 (15)	361>165 (12)		
1	8	Cortisone	6	10.96	10.99			531>168 (15)	441>160 (18)		
2	1	5β-Androstane-3β,17β-diol	1	6.76				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	1	5α-Androstane-3α,17β-diol	1	6.89				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	1	5β-Androstane-3α,17β-diol	1	6.97				421>255 (9)	346>256 (6)	346>241 (6)	331>241 (6)
2	2	5-Androstene-3β,7α,17β-triol	1	7.29				432>327 (12)	432>233 (24)	432>209 (15)	
2	3	5α-Androstane-3β,17β-diol	1	7.66				421>255 (12)	346>241 (15)	331>241 (6)	
2,3	3	Androstenediol	1	7.70				344>239 (15)	329>239 (9)	329>197 (18)	
2	4	Epietiocholanolone	1	7.95				360>270 (9)	270>213 (9)	270>157 (21)	
2,3	4	Androsterone	1	8.05				360>270 (9)	270>213 (9)	270>157 (21)	
2	4	Etiocholanolone	1	8.13				360>270 (9)	270>213 (9)	270>157 (21)	
2	4	5-Androstene-3β,7β,17β-triol	1	8.17				432>327 (15)	432>233 (21)	432>209 (18)	
2	5	7α-Hydroxy-DHEA	1	8.34				387>247 (15)	387>219 (30)		

Table 1., continued.

Injection	MRM-AW	Steroid	IS[a]	Retention time [min]				MRM transition (collision energy [V])			
				peak 1	peak 2	peak 3	peak 4	MRM transition 1	MRM transition 2	MRM transition 3	MRM transition 4
2,3	5	5α-Pregnane-3α,20α-diol	4(1[a])	8.41				269>187 (12)	269>161 (12)	269>105 (30)	
2,3	5	5β-Pregnane-3α,20α-diol	4(1[a])	8.46				269>187 (12)	269>161 (12)	269>105 (30)	
1	1	D6-DHEA sulfate (IS1, conjugates)		8.61				364>274 (9)			
2,3	6	D6-DHEA (IS2)		8.61				364>274 (9)			
2	6	Estradiol	1	8.61				416>285 (15)	416>326 (6)	285>205 (15)	
2,3	6	Epiandrosterone	1	8.63				360>270 (9)	360>84 (18)	360>82 (21)	
2,3	6	Dehydroepindrosterone (DHEA)	1	8.64				358>84 (18)	268>82 (21)	260>213 (6)	
2,3	6	5-Androsten-3β,16α,17β-triol	1	8.65				432>327 (15)	432>239 (15)	329>239 (9)	
2	6,7	Epitestosterone	2	8.70	8.81			389>268 (9)	389>137 (12)		
2	7	5α-Dihydrotestosterone	2	8.78	8.79			391>360 (12)	391>286 (6)	286>254 (6)	
2,3	7	Epipregnanolone	4(1[a])	8.86				388>298 (15)	388>173 (18)	388>70 (18)	
2,3	7	5α-Pregnane-3β,20α-diol	4(1[a])	8.93				449>117 (12)			
2	7	20α-Dihydropregnenolone	3(1[a])	8.93				372>117 (18)	332>117 (12)		
2	7	7β-Hydroxy-DHEA	1	8.95				387>247 (15)	387>219 (30)		
2,3	7	Allopregnanolone	4(1[a])	8.96				388>298 (15)	388>173 (18)	388>70 (18)	
2	7	Testosterone	2	8.98	9.12			389>268 (9)	389>137 (12)	389>125 (9)	
2,3	7	Pregnanolone	4(1[a])	9.03				388>298 (15)	388>173 (18)	388>70 (18)	
2	8	17α-Hydroxypregnenolone	3(1[a])	9.24				474>294 (9)	474>225 (12)	474>157 (21)	
2,3	9	Estriol	1	9.41				504>311 (18)	345>255 (12)		
2,3	9	Isopregnanolone	4(1[a])	9.42				388>173 (21)	388>107 (27)	388>70 (24)	
2	9	Pregnenolone	4(1[a])	9.43				402>239 (12)	312>239 (9)	239>157 (18)	
2,3	10	5β,20α-Tetrahydroprogesterone	4(1[a])	9.53	9.55			303>288 (9)	303>159 (27)		
2	10	5α-Androstane-3,17-dione	1	9.59	9.61			315>83 (27)	315>244 (21)		
2	10	16α-Hydroxypregnenolone	3(1[a])	9.61				474>156 (27)			
2	10	16α-Hydroxytestosterone	2	9.65	9.74			477>153 (18)			
2	10	Androstenedione	2	9.77	9.88			344>313 (9)	344>137 (24)	344>125 (15)	
2	10	5α,20α-Tetrahydroprogesterone	4(1[a])	9.80	9.82			303>288 (9)	303>159 (27)		
2	11	7-oxo-DHEA	1	9.99				401>148 (18)	386>235 (30)		
2	11	20α-Dihydroprogesterone	4(1[a])	9.99	10.10			417>117 (12)	301>286 (9)	301>138 (15)	
2	11	5β-Dihydroprogesterone	4(1[a])	10.01	10.03			343>259 (18)	343>244 (33)		
2	12	D8-17α-Hydroxyprogesterone (IS3)		10.14	10.21			437>377 (18)			
2	12	17α-Hydroxyprogesterone	3(1[a])	10.18	10.24			429>370 (18)	429>170 (12)		
2	12	5α-Dihydroprogesterone	4(1[a])	10.27	10.29			343>244 (24)	343>272 (18)	288>159 (18)	
2	13	D9-Progesterone (IS4)		10.41	10.49			381>350 (9)			
2	13	Progesterone	4(1[a])	10.45	10.55			372>341 (9)	341>269 (12)		
2	13	16α-Hydroxyprogesterone	3(1[a])	10.53	10.61			429>370 (15)	429>156 (18)	156>73 (15)	

[a]D6-DHEA sulfate (IS1) was used as internal standard for conjugated steroids.

Table 2. List of abbreviations for endogenous steroids, linearity of the response and linear dynamic range.

ID	Abbreviation	Steroid	Correlation coefficient *r*	Linear dynamic range [pg injected]
1	Preg	Pregnenolone	0.9995	0.077-2000
2	Preg17	17α-Hydroxypregnenolone	0.9996	0.12-2000
3	Preg16α	16α-Hydroxypregnenolone	0.9997	0.12-2000
4	DHPreg20α	20α-Dihydropregnenolone	0.9991	0.12-2000
5	DHEA	Dehydroepiandrosterone	0.9978	07.08.2000
6	DHEA7α	7α-Hydroxy-DHEA	0.9995	0.12-2000
7	DHEA7o	7-oxo-DHEA	0.9952	0.49-2000
8	DHEA7β	7β-Hydroxy-DHEA	0.9987	0.49-2000
9	5-Adiol	5-Androstene-3β, 17β-diol	0.9979	0.49-2000
10	AT7α	5-Androstene-3β,7α,17β-triol	0.9999	0.49-2000
11	AT7β	5-Androstene-3β,7β,17β-triol	0.9993	0.12-2000
12	AT16α	5-Androstene-3β,16α,17β-triol	0.9985	0.49-2000
13	P	Progesterone	0.9998	0.12-10000
14	P17	17α-Hydroxyprogesterone	0.9997	0.12-2000
15	DHP17α20α	17α,20α-Dihydroxy-4-pregnene-3-one	0.9957	0.12-10000
16	P16α	16α-Hydroxyprogesterone	0.9998	0.12-2000
17	DHP20α	20α-Dihydroprogesterone	0.9997	0.49-2000
18	A4	Androstenedione	0.9988	0.49-2000
19	T	Testosterone	0.9998	2.0-2000
20	T16α	16α-Hydroxytestosterone	0.9997	2.0-2000
21	DHT5α	5α-Dihydrotestosterone	0.9994	0.49-2000
22	E1	Estrone	0.9995	7.8-10000
23	E2	Estradiol	0.9996	0.12-2000
24	E3	Estriol	0.9999	7.8-10000
25	DHP5α	5α-Dihydroprogesterone	0.9995	0.12-10000
26	THP3α5α	Allopregnanolone	0.9996	0.12-2000
27	THP3β5α	Isopregnanolone	0.9995	0.49-2000
28	DHP5β	5β-Dihydroprogesterone	0.9986	7.8-10000
29	THP3α5β	Pregnanolone	0.9995	0.12-2000
30	THP3β5β	Epipregnanolone	0.9996	0.12-2000
31	THP5α20α	5α,20α-Tetrahydroprogesterone	0.9995	0.12-2000
32	PD3α5α20α	5α-Pregnane-3α,20α-diol	0.9995	0.12-10000
33	PD3β5α20α	5α-Pregnane-3β,20α-diol	0.9987	7.8-10000
34	THP5β20α	5β,20α-Tetrahydroprogesterone	0.9999	0.12-2000
35	PD3α5β20α	5β-Pregnane-3α,20α-diol	0.9995	0.12-2000
36	PD3β5β20α	5β-Pregnane-3β,20α-diol	0.9997	0.49-10000
37	PD3α5α17	17α-Hydroxyallopregnanolone	0.9994	0.49-2000
38	PD3α5β17	17α-Hydroxypregnanolone	0.9995	0.49-2000
39	PT3α5α17α20α	5α-Pregnane-3α,17α,20α-triol	0.9981	0.12-10000
40	PT3β5α17α20α	5α-Pregnane-3β,17α,20α-triol	0.9977	0.12-10000
41	PT3α5β17α20α	5β-Pregnane-3α,17α,20α-triol	0.9982	0.12-10000
42	DHA5α	5α-Androstane-3,17-dione	0.9993	0.12-10000
43	THA3α5α	Androsterone	0.9987	0.12-2000
44	THA3β5α	Epiandrosterone	0.9991	2.0-2000
45	THA3α5β	Etiocholanolone	0.9994	0.12-2000
46	AD3α5α17β	5α-Androstane-3α,17β-diol	0.9996	0.12-2000
47	AD3β5α17β	5α-Androstane-3β,17β-diol	0.9989	0.12-2000
48	AD3α5β17β	5α-Androstane-3α,17β-diol	0.9996	0.12-2000
49	F	Cortisol	0.9991	31-10000
50	E	Cortisone	0.9972	125-10000
51	B	Corticosterone	0.9987	7.8-10000
52	DOF	21-Deoxycortisol	0.9991	0.49-2000

Table 2., continued.

ID	Abbreviation	Steroid	Correlation coefficient *r*	Linear dynamic range [pg injected]
53	DOC	11-Deoxycorticosterone	0.9999	2-10000
54	THB3α5α	3α,5α-Tetrahydrocorticosterone	0.9995	0.12-10000
55	THB3α5β	3α,5β-Tetrahydrocorticosterone	0.999	0.49-10000
56	11OHA4	11β-Hydroxyandrostenedione	0.9978	0.49-10000
57	THA3α5α11β	11β-Hydroxyandrosterone	0.9998	0.12-2000
58	THA3β5α11β	11β-Hydroxyepiandrosterone	0.9983	0.12-2000
59	THA3α5β11β	11β-Hydroxyetiocholanolone	0.9999	0.12-2000
60	PregC	Pregnenolone sulfate	0.9994	0.077-2000
61	Preg17C	17α-Hydroxypregnenolone sulfate	0.9996	0.12-2000
62	DHPreg20αC	20α-Dihydropregnenolone sulfate	0.9991	0.12-2000
63	DHEAC	DHEA sulfate	0.998	7.8-2000[a]
64	5-AdiolC	Androstenediol sulfate	0.9981	0.49-2000
65	AT16αC	5-Androstene-3β,16α,17β-triol sulfate	0.9986	0.49-2000
66	DHP17α20αC	Conjugated 17α,20α-dihydroxy-4-pregnen-3-one	0.9945	0.12-10000
67	DHP20αC	Conjugated 20α-dihydroprogesterone	0.9997	0.49-2000
68	TC	Conjugated testosterone	0.9993	2.0-2000
69	EpiTC	Conjugated epitestosterone	0.9997	0.49-2000
70	E1C	Estrone sulfate	0.9993	7.8-10000
71	E2C	Estradiol sulfate	0.9991	0.12-2000
72	E3C	Estriol sulfate	0.9994	7.8-10000
73	THP3α5αC	Allopregnanolone sulfate	0.9995	0.12-2000
74	THP3β5αC	Isopregnanolone sulfate	0.9997	0.49-2000
75	THP3α5βC	Conjugated pregnanolone	0.9994	0.12-2000
76	THP3β5βC	Conjugated epipregnanolone	0.9994	0.12-2000
77	THP5α20αC	Conjugated 5α,20α-tetrahydroprogesterone	0.9986	0.12-2000
78	PD3α5α20αC	Conjugated 5α-pregnane-3α,20α-diol	0.9994	0.12-10000
79	PD3β5α20αC	Conjugated 5α-pregnane-3β,20α-diol	0.9981	7.8-10000
80	THP5β20αC	Conjugated 5β,20α-tetrahydroprogesterone	0.9998	0.12-2000
81	PD3α5β20αC	Conjugated 5β-pregnane-3α,20α-diol	0.9995	0.12-2000
82	PD3β5β20αC	Conjugated 5β-pregnane-3β,20α-diol	0.9994	0.49-10000
83	PD3α5α17C	17α-Hydroxyallopregnanolone sulfate	0.9994	0.49-2000
84	PD3α5β17C	Conjugated 17α-hydroxypregnanolone	0.9996	0.49-2000
85	PT3α5α17α20α	5α-Pregnane-3α,17α,20α-triol	0.9981	0.12-10000
86	PT3β5α17α20α	5α-Pregnane-3β,17α,20α-triol	0.9977	0.12-10000
87	PT3α5β17α20α	5β-Pregnane-3α,17α,20α-triol	0.9982	0.12-10000
88	THA3α5αC	Androsterone sulfate	0.9987	0.12-2000[a]
89	THA3β5αC	Epiandrosterone sulfate	0.9993	2.0-2000[a]
90	THA3α5βC	Etiocholanolone sulfate	0.9995	0.12-2000
91	THA3β5βC	Epietiocholanolone sulfate	0.9992	0.49-2000
92	AD3α5α17βC	Conjugated 5α-androstane-3α,17β-diol	0.9994	0.12-2000
93	AD3β5α17βC	Conjugated 5α-androstane-3β,17β-diol	0.9996	0.12-2000
94	AD3α5β17βC	Conjugated 5β-androstane-3α,17β-diol	0.9992	0.12-10000
95	AD3β5β17βC	Conjugated 5β-androstane-3β,17β-diol	0.9992	0.12-10000
96	THB3α5αC	Conjugated 3α,5α-tetrahydrocorticosterone	0.9994	0.12-10000
97	THB3α5βC	Conjugated 3α,5β-tetrahydrocorticosterone	0.9994	0.12-10000
98	THA3α5α11βC	11β-Hydroxyandrosterone sulfate	0.998	0.12-2000
99	THA3β5α11βC	11β-Hydroxyepiandrosterone sulfate	0.9985	0.12-2000
100	THA3α5β11βC	11β-Hydroxyetiocholanolone sulfate	0.9982	0.12-2000

[a]Additional application of 0.2 µl sample (third injection aliquot) besides of the usual 2 µl injection volume (for unconjugated steroids and most steroid conjugates – first and second injection aliquots) to quantify two steroid conjugates above the upper limit of the linear dynamic range.

Independent analytical methods used for accuracy testing

To compare some results of the present method, we measured 47 analytes using our previously published GC-MS method (Hill *et al.* 2010b), 6 analytes by our LC-MS/MS method (Vitku *et al.* 2016) and cortisol was also measured by radioimmunoassay from Immunotech (Marseille, France).

Method performance characteristics

Calibration curve and linearity of the response

The calibration was performed in charcoal-stripped serum. The analytes were quantified using calibration curves based on known concentrations in the mixtures of analyzed standards with constant level of ISs. We used a 9-point logarithmic calibration curve. The values were corrected for procedural losses according to yields of ISs. The use of ISs for individual steroids is shown in Table 1. The amount of each steroid injected from the calibration samples into the GC-corresponded to amount of 10 ng, 2 ng, 500 pg, 125 pg, 31.2 pg, 7.81 pg, 1.95 pg, 488 fg and 122 fg. The calibration curves were constructed by plotting the logarithm of response factor (analyte area/internal standard area) against the logarithm of concentration of the calibration (external) standard to cover the large concentration differences for circulating steroids in different physiological and pathophysiological situations and even more explicit contrasts between unconjugated steroids and their conjugated counterparts at appropriate number of calibration points. This arrangement also provided equal weights for individual calibration points in the logarithmic calibration curve and therefore the use of weighted regression model was not necessary to apply. The assay acceptance criterion for each back-calculated standard concentration was set 15 % deviation from the nominal value.

Precision

The method precision (intra-assay, within-day) and intermediate precision (inter-assay, between-day) was based on the concentrations of each analyte. Regarding gender differences in the levels of testosterone and its metabolites, elevated levels of progesterone and its metabolites in the luteal menstrual phase and excessive levels of numerous steroids in serum from pregnant women and in umbilical cord serum, the precision was evaluated separately in pooled sera for adult men, women in the follicular menstrual phase, luteal menstrual phase, pregnant women at labor and for mixed umbilical cord sera at labor. The method precision was calculated from steroid concentrations in six identical samples, which were prepared from the aforementioned pools within one batch prepared on the same day. Similarly, intermediate precision was estimated from the steroid concentrations in six identical samples but these were prepared in separate batches on different days. The precision was expressed as percent of relative standard deviation (RSD).

Recovery

The recovery indicates the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method (Bioanalytical Method Validation 2018). In the present method, the recovery was determined by spiking charcoal-stripped serum with three concentrations of the individual analytes taking into account steroid levels in the corresponding pools. The recovery experiments were performed by comparing the analytical results of extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction (Bioanalytical Method Validation 2018) in replicates from four independent runs.

Accuracy

Accuracy was expressed as relative error of the measured concentration of each steroid with respect to its true spiked concentration (% bias). The accuracy testing was performed for three different concentrations of analytes dissolved in charcoal-stripped plasma, which were close to their physiological levels. The bias was tested in both intra- and inter-day experiments. The corresponding samples for accuracy testing were processed in the same way as the calibration and unknown samples (see section ***Stock solutions, calibration standards, and quality control samples*** and section ***Sample preparation***). The bias less then ±15 % was met for all analytes in all tested concentrations in both intra- and inter-day experiments. The analytes, which did not meet these criteria, were not included in this method.

Furthermore, we compared our present GC-MS/MS method with our previous GC-MS method for 45 steroids in samples covering all types of human sera (Table S1) and also tested an agreement of six common steroids (pregnenolone, 17α-hydroxypregenolone, DHEA, androstenedione, testosterone and cortisol)

measured by our present method with the LC-MS/MS method (Hill *et al.* 2010b) in samples mostly consisting of the women in follicular menstrual phase but there were also some women in the luteal phase, postmenopausal women and men (Table S2). Besides the LC-MS/MS and GC-MS/MS, the cortisol was also evaluated using an RIA kit from Immunotech (Marseille, France). The comparison was performed using Bland-Altman procedure (Bland and Altman 1986) and a robust Passing Bablok regression with the use of R library "mcr" (Manuilova *et al.* 2014).

Limit of detection and limit of quantification

Because the baseline noise was accessible for all analytes in all matrixes (pools), the limit of detection (LOD) and limit of quantification (LOQ) were estimated using charcoal stripped plasma spiked with steroids in three levels covering gender differences and distinct physiological status in women. The LOD was calculated as 3.3 times of the baseline noise using charcoal stripped plasma vs. charcoal stripped plasma spiked with steroid on the first level with lowest concentration of analyte.

The lowest nonzero standard on the calibration curve defined the LOQ. The satisfactory analyte response at the LOQ in the present method was at least five times the analyte response of the zero calibrator and the satisfactory bias at the LOQ was at most ±20 % of nominal concentration. Similarly, the satisfactory imprecision at the LOQ was at most ±20 % RSD. For this purpose, we tested the replicates prepared in six runs (Bioanalytical Method Validation 2018). The determination of signal to noise ratios (S/N) for the calculation of LOD was completed using a functionality in the Shimadzu software GCMSsolution Version 4.20, which was a component of our GC-MS/MS system.

Efficiency of methanolysis and stability of non-deuterated and deuterated steroids

Unfortunately, the external standards for steroid sulfates and glucuronides are not available for the full spectrum of the quantified steroid conjugates. Therefore, we have tested the efficiency of methanolysis for only seven sulfated non-deuterated steroids (6 sulfates and one disulfate) and D6-dehydroepiandrosterone sulfate (D6-DHEA). The procedure was as follows. The 100 µl or 10 µl aliquots of the stock solution of unconjugated steroid and sulfated steroid were administered into the glass extraction tubes and dried under the flow of nitrogen. Then 20 µl of methanol was added and the solution was shortly mixed. The addition of 1 ml of charcoal-stripped mixed human plasma followed and the solution was then mixed for 1 min. The obtained samples for each steroid or steroid sulfate were processed in the same way as the calibration and unknown samples (see section ***Stock solutions, calibration standards, and quality control samples*** and section ***Sample preparation***). The responses (areas under the peak) for polar and non-polar phases after diethyl ether extraction for individual unconjugated steroids, corresponding steroid conjugates and for internal standard (D6-DHEA) were used to calculate extraction efficiency for unconjugated steroids and sulfated steroids, as well as the efficiency of methanolysis in sulfated steroids.

The analysis of chemical stability during the methanolysis for unconjugated steroids was based on the comparison of calibration samples for unconjugated analytes, which were exposed to methanolysis procedure with the same samples, which did not undergo this route.

Terminology of steroid polar conjugates

Concerning the terminology of the steroid polar conjugates used here, the term steroid sulfate was used in the case of the dominance of 3α/β-monosulfate over other forms of steroid conjugates, while the term conjugated steroid was used in the case of comparable amounts of conjugate forms (sulfates, disulfates, and glucuronides). This terminology was based on the relevant literature, with appropriate citations for each steroid as follows: Preg sulfate (Brochu and Belanger 1987, Sanchez-Guijo *et al.* 2015), DHPreg20α sulfate, dehydroepiandrosterone (DHEA) sulfate (Brochu *et al.* 1987, Labrie *et al.* 1997, Sanchez-Guijo *et al.* 2015), 5-Adiol sulfate (Labrie *et al.* 1997, Sanchez-Guijo *et al.* 2015), THP3α5α sulfate, THP3β5α sulfate (Abu-Hayyeh *et al.* 2013), conjugated THP3α5β (sulfate + glucuronide) (Meng *et al.* 1997), PD5α3β20α sulfate (3β,20α-disulfate + 3β-sulfate) (Meng *et al.* 1997), conjugated PD3α5β20α (3β,20α-disulfate + 3β-sulfate + glucuronide) (Meng *et al.* 1997), THA3α5α sulfate (Labrie *et al.* 1997, Sanchez-Guijo *et al.* 2015), THA3β5α sulfate (Labrie *et al.* 1997, Sanchez-Guijo *et al.* 2015), THA sulfate3α5β (Tokushige *et al.* 2013), THA sulfate 3β5β, conjugated (glucuronide + sulfate) (Labrie *et al.* 1997), and conjugated AD3β5α17β (sulfate + glucuronide) (Labrie *et al.* 1997).

Results and Discussion

In total, the levels of 100 analytes (58 unconjugated steroids and 42 steroid conjugates) were quantified in samples of pooled sera from groups of adult men, women in the follicular menstrual phase, women in the luteal menstrual phase, pregnant women at labor and in umbilical cord serum at labor (Tables 2 and 3). The steroid metabolome in the maternal circulation included the levels of C21 Δ^5 steroids, C19 Δ^5 steroids, C21 Δ^4 steroids, C19 Δ^4 steroids, estrogens, C21 and C19 5α/β-reduced steroids, 7α-hydroxy-, 16α-hydroxy-, 7β-hydroxy- and 7-oxo-derivatives of C19 Δ^5 steroids, and 20α-dihydro-metabolites of C21 steroids (20α-dihydro-pregnanes) (Table 2). Figures 3-6 show a comparison of the chromatograms for calibration samples and samples prepared from five pools of human serum and recorded on quantification MRM transitions for unconjugated steroids, which are less abundant then their conjugated counterparts (Table 3).

Validation parameters

Linearity of the response

Sufficient linearity was found for broad range of concentrations (Table 2). The 15 % deviation from the nominal value for each back-calculated standard concentration as the criterion of assay acceptance was not exceeded in any case.

Precision

As expected, the higher precision was typically obtained for more abundant steroids. For instance, better results were obtained for C19 steroids in non-pregnant subjects but for C21 steroids in pregnant women and in mixed umbilical serum. Higher precision was achieved for more abundant steroid conjugates when compared with their less abundant unconjugated counterparts. The results for T, DHT5α and 5-Adiol were generally better in pooled serum from adult men when compared with other groups. As concerns the accessibility of hydroxy-group for derivatization, the 11β-hydroxy-steroids showed lower precision when compared with their 11-deoxy-counterparts due to difficult accessibility of 11β-hydroxy-group for the sylilating agent.

If the intra- and/or inter-assay exceeded the 15 % RSD in some of the tested pooled samples, the validation in this biological material was considered as unsatisfactory. For instance, the levels of several reduced 5β-reduced C21 steroids are insufficient to quantify these analytes out of pregnancy. However, in a nutshell, most analytes may be quantified in all investigated matrixes (Table 3).

Recovery

In general, the additions of steroids for the computation of recovery were derived from steroid levels in the pooled sample. In two steroid sulfates such as DHEA sulfate and THA3α5αC, the samples for recovery were diluted to be within the LDR (Table S3). As expected, the recovery rates differed according to the steroid polarity. On the one hand, the diethyl-ether extraction step should be more favorable for the less polar steroids but on the other hand, partitioning between the methanol-water mixture and pentane should be less efficient for the steroids with low polarity. When testing the recovery, we found lower values for less polar steroids such as 5α/β reduced C21 steroids but high values for the polar ones such as cortisol. The number of hydroxy-groups positively correlates with the recovery rate (for instance allopregnanolone vs. 5α-pregnane-3α,20α-diol or allopregnanolone vs. 17-hydroxyallopregnanolone). The 5α/β-reduced steroids showed lower recovery rates in comparison with their unsaturated counterparts (for instance 5α-dihydroprogesterone vs. progesterone or 5α-dihydro-testosterone vs. testosterone). The C19 steroids generally exhibit higher recovery rates in comparison with their C21 analogues (for instance androsterone vs. allopregnanolone).

Accuracy

The accuracy test was not carried out if the intra- and/or inter assay for precision exceeded the 15 % RSD (Table 3). When the precision testing was acceptable, the bias less then ±15 % was met for all analytes in all tested concentrations in both intra- and inter-day experiments (Table S4).

Stability tests

A stability test after three freeze and thaw cycles did not show statistically significant differences. There were also no significant differences found for a temperature stability test after leaving the sample for one day at room temperature, a 3-day post-preparative stability test for steroids after derivatization at room temperature, or for one-month stability test for the stock solutions of analytes.

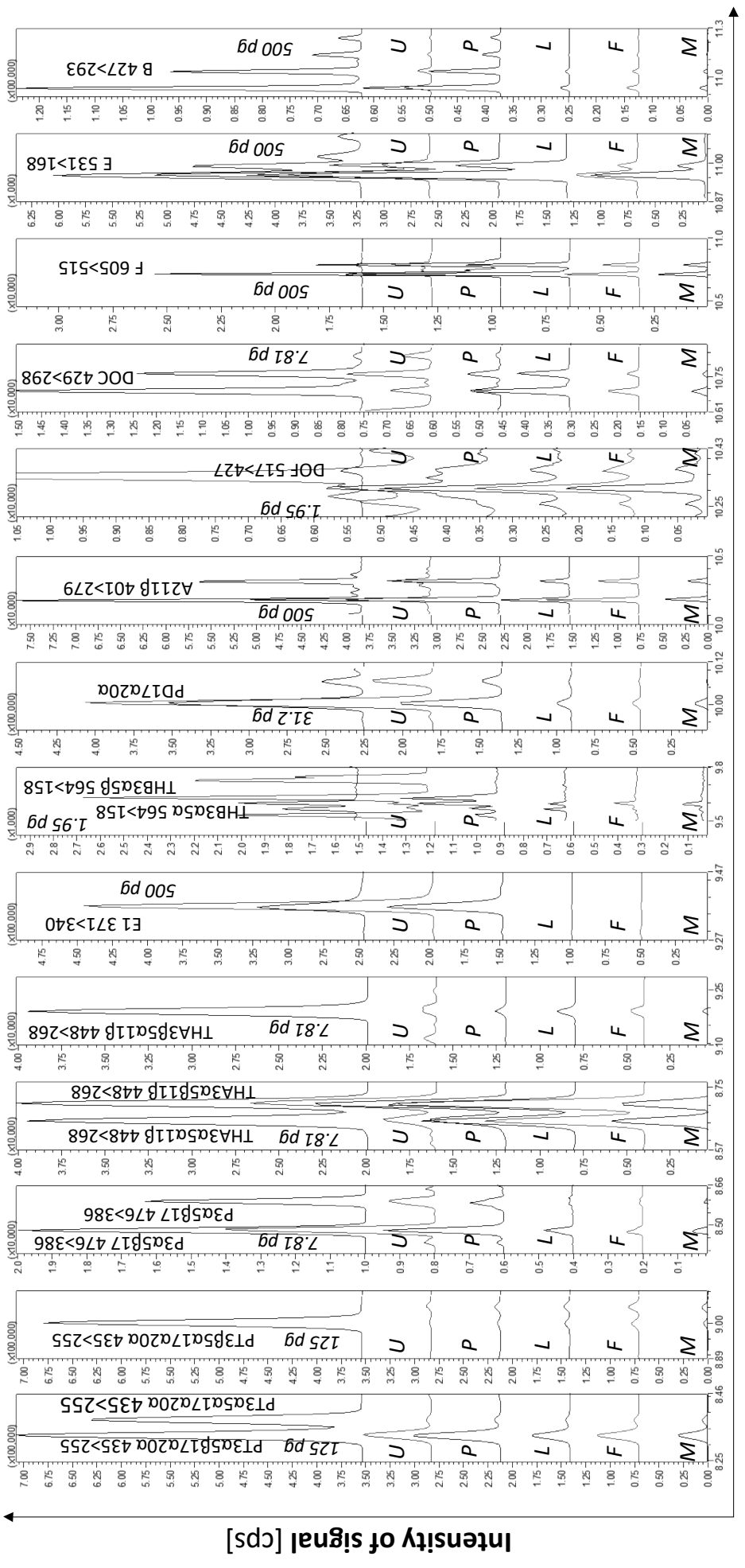

Fig. 3. Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M – males, F – follicular menstrual phase, L – luteal menstrual phase, P – pregnant women at labor, U – mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.

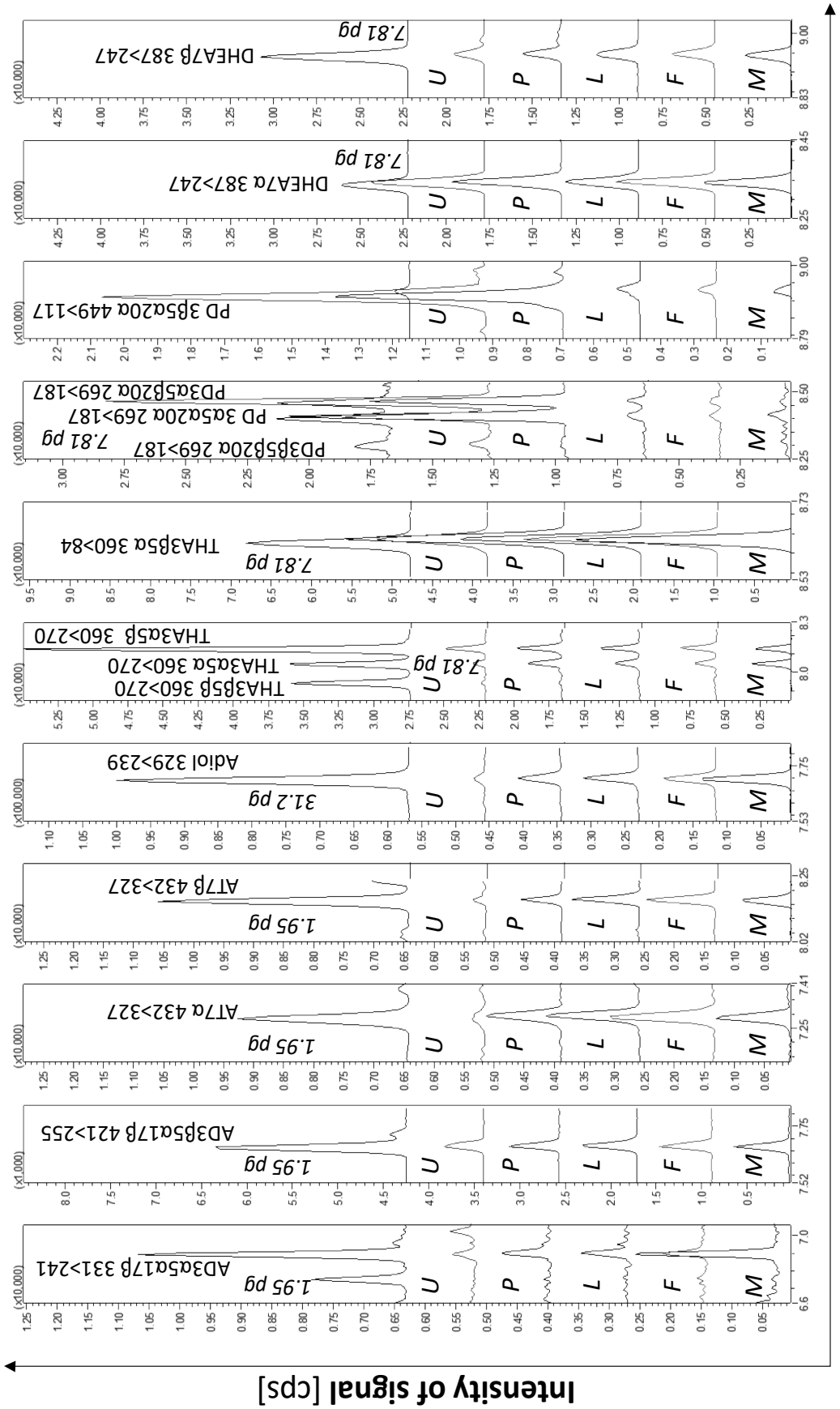

Fig. 4. Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M – males, F – follicular menstrual phase, L – luteal menstrual phase, P – pregnant women at labor, U – mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.

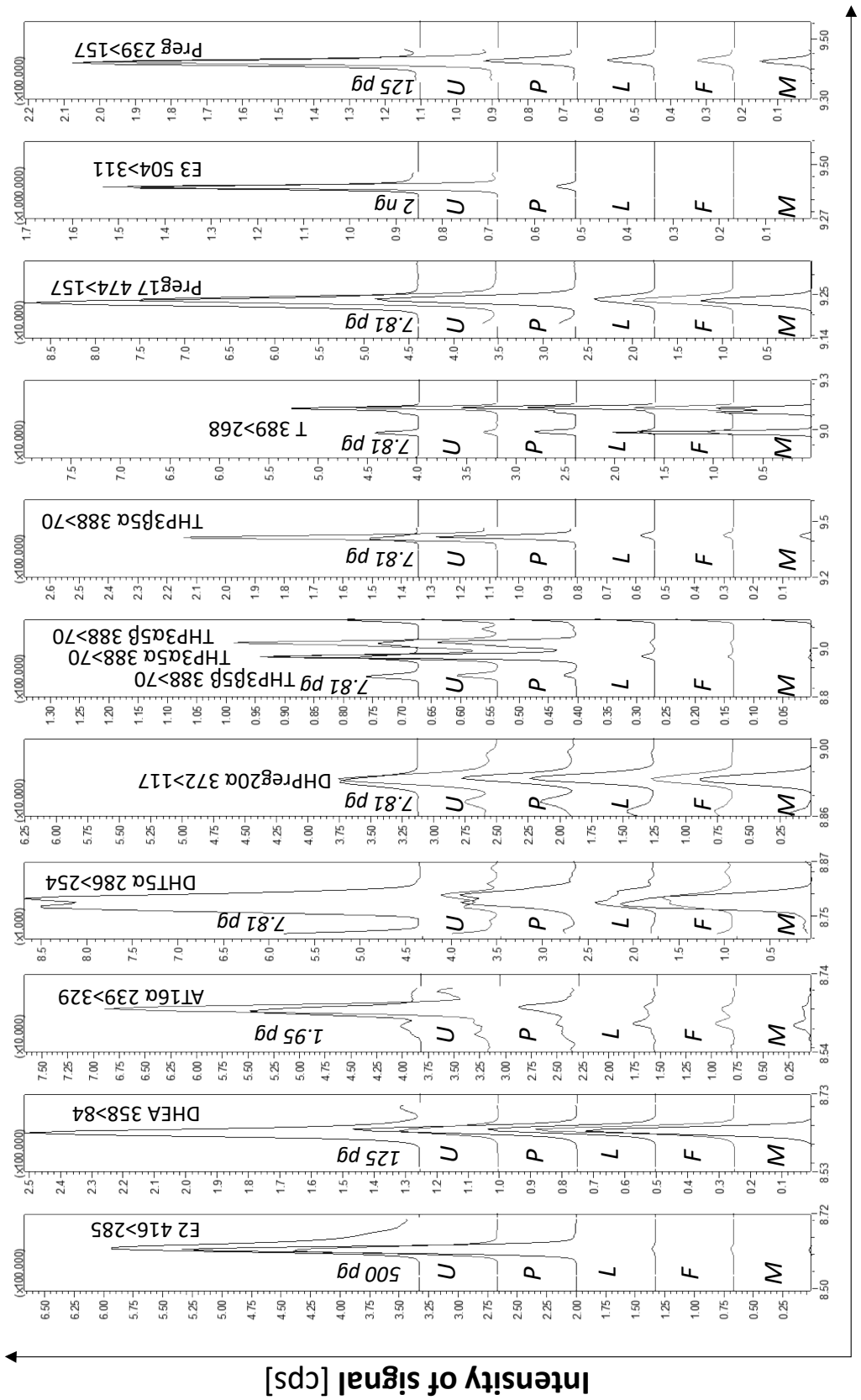

Fig. 5. Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M – males, F – follicular menstrual phase, L – luteal menstrual phase, P – pregnant women at labor, U – mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.

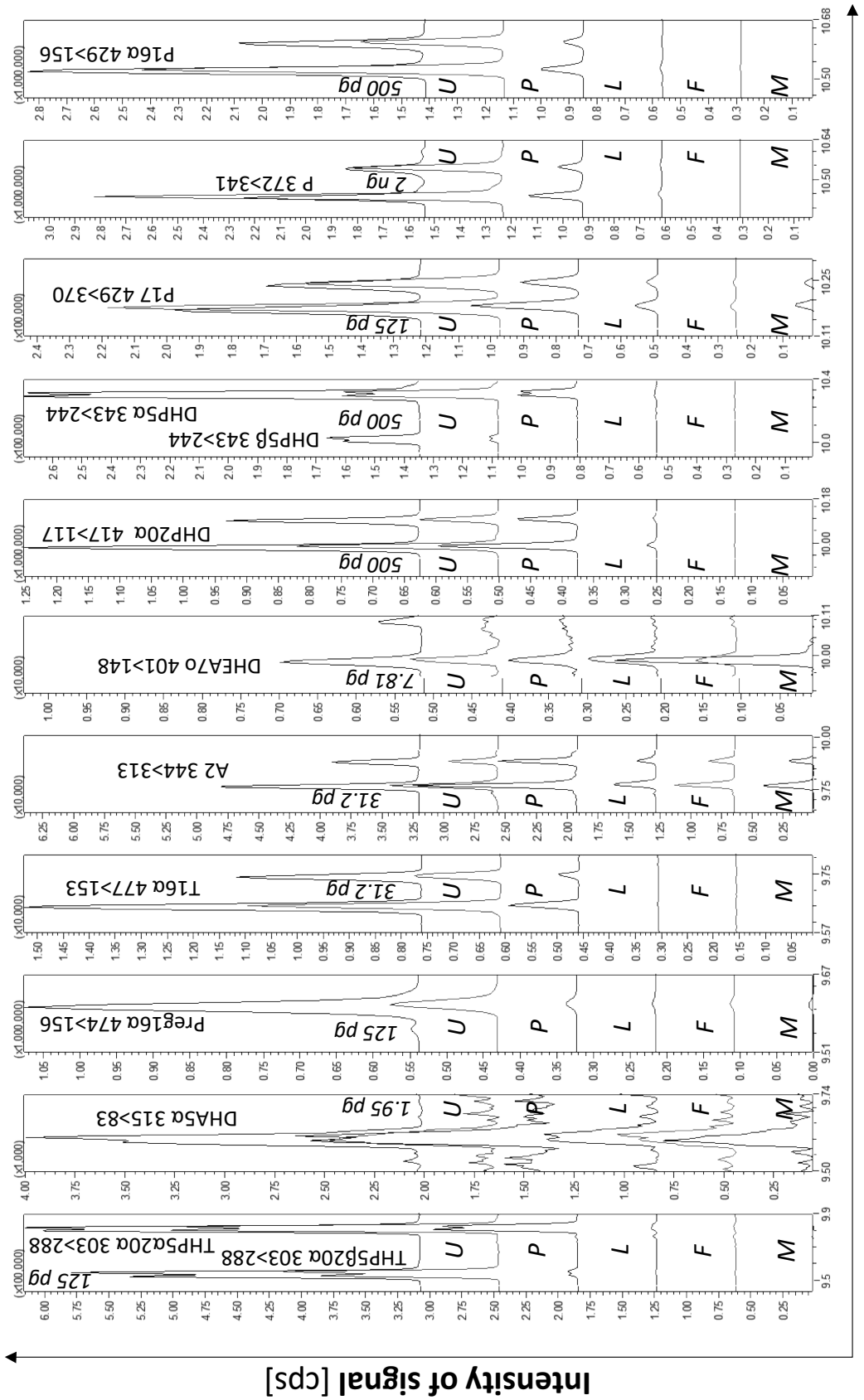

Fig. 6. Comparison of the chromatograms for calibration samples prepared from the charcoal stripped plasma and added steroids and samples of unconjugated steroids prepared from different pools of human serum and recorded on quantification MRM transitions. Numbers in embedded tables represent amounts of derivatized steroids in calibration samples (pg) injected to the GC-MS/MS system, M – males, F – follicular menstrual phase, L – luteal menstrual phase, P – pregnant women at labor, U – mixed umbilical serum at labor. Abbreviations of steroids are explained in Table 2.

Table 3. Sensitivity, Intra-assay and Inter-assay relative standard deviations (RSDs) for GC-MS/MS analysis of endogenous unconjugated steroids in human serum.

ID	Steroid	LOD [pg]	LOQ [pg] (bias + precision at LOQ)	Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
				Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]
1	Preg	0.02	2(5.8%,18%)	32/5.1	1.3/12	53/8.4	2.5/9.4	58/9.2	2/13	110/18	0.91/7.9	470/74	0.91/7.9
2	Preg17	0.05	0.5(-5.8%,10%)	80/12	1.5/11	86/13	0.98/9.5	56/8.4	1.2/7.4	160/24	1.7/6.4	220/33	0.87/6.6
3	Preg16α	0.009	0.5(13%,3.3%)	2.9/0.43	4.8/8.5	2.9/0.43	2.8/4.5	2.2/0.33	8.6/8.5	5.4/0.81	3.5/5.9	47/7.1	1.1/7.1
4	DHPreg20α	0.03	0.5(-6%,11%)	15/2.3	4.3/9.8	22/3.4	6.1/9.4	27/4.2	1.2/10	25/4	2.9/5.4	32/5.1	2.9/5.9
5	DHEA	0.008	2(9.5%,6%)	58/10	1.4/6.8	86/15	1.4/4.7	69/12	1.6/3.8	100/18	1.8/4.7	44/7.7	2.6/5.1
6	DHEA7α	0.02	0.5(-1.7%,11%)	7.9/1.3	1.6/8.3	9.1/1.5	2.8/4	5.8/0.96	4.3/6.3	5.5/0.91	1.3/7.4	12/2	1.8/6.2
7	DHEA7o	0.09	0.5(7.7%,11%)	6.6/1.1	6.7/11	2.5/0.41	13/9.9	2.4/0.39	7.7/12	3.2/0.53	8.4/15	4.8/0.79	7.2/7.9
8	DHEA7β	0.03	0.5(3.2%,13%)	2.9/0.48	7.1/14	1.5/0.25	4/14	2.4/0.4	8.5/13	1/0.17	5.2/7.3	2/0.33	7.5/9.7
9	5-Adiol	0.1	2(0.61%,10%)	15/2.5	1.4/6.7	13/2.3	2/8	11/1.9	2.9/10	8.7/1.5	2.6/7	2.6/0.44	6.5/6.4
10	AT7α	0.02	0.5(15%,4.9%)	2.3/0.37	2.7/10	2.7/0.44	2.4/6.8	1.7/0.28	5.2/8.9	0.6/0.098	3.4/10	---	15/11
11	AT7β	0.02	0.5(13%,8.4%)	1.9/0.31	8.6/12	2.1/0.35	5.3/5.7	1.5/0.25	12/7.3	0.42/0.068	6.9/11	---	7.7/13
12	AT16α	0.04	0.5(-2.6%,20%)	3.1/0.51	13/12	2.8/0.45	9.9/13	3.1/0.51	12/13	4.9/0.8	13/12	19/3.1	5.5/11
13	P	30	0.5(-2.1%,12%)	1.5/0.24	6.3/11	1.6/0.25	13/11	75/12	2.2/14	2000/320	0.61/8	14000/2300	0.53/7.5
14	P17	0.1	0.5(5.1%,11%)	18/2.8	4.3/9.1	7.3/1.1	4.4/5.4	21/3.2	3.7/14	120/18	1.3/8.9	650/99	0.79/8.4
15	DHP17α20α	1	0.5(6.3%,7.6%)	10/1.5	1.3/8.6	4.5/0.68	1.7/11	6.6/1	1.7/4.4	56/8.4	1.3/15	170/26	0.74/10
16	P16α	0.02	0.5(7.2%,3.6%)	5/0.76	3.3/11	3.2/0.48	1.5/7.6	6.3/0.96	3/6.2	130/19	0.64/5.9	920/140	0.58/8.4
17	DHP20α	0.02	0.5(13%,8.6%)	1.3/0.21	6.9/8.9	1.8/0.29	3.1/8.5	31/4.9	0.81/11	580/92	0.6/6	630/99	0.69/6.7
18	A4	0.09	2(-4.3%,9.8%)	15/2.6	0.88/11	15/2.7	3.2/7.9	13/2.3	4.9/14	49/8.6	2.5/8.2	86/15	4.4/7.7
19	T	0.02	2(18%,1.6%)	86/15	2.2/8	8.1/1.4	10/10	5.8/1	6.2/11	19/3.3	5.8/6.9	4.3/0.74	12/6.8
20	T16α	0.3	2(-5.8%,15%)	---	---	---	---	---	---	26/4.2	2.9/13	67/11	4.6/8.2
21	DHT5α	0.04	0.5(9.5%,5.8%)	8.7/1.5	6.4/9.1	3/0.51	9/8.6	2.9/0.5	15/8.5	3.4/0.58	4.3/9.8	0.81/0.14	14/15
22	E1	0.07	0.5(-1.3%,8.6%)	0.86/0.16	4.8/14	1.3/0.24	7.8/10	1.4/0.26	4.8/11	260/48	6.6/9	650/120	0.4/7.5
23	E2	0.02	0.5(-2.6%,11%)	0.54/0.1	7.7/15	2.1/0.38	5.3/13	2.3/0.42	8.9/6.5	370/68	0.41/9	180/33	0.83/8.1
24	E3	0.05	2(-5.8%,8.2%)	---	---	---	---	---	---	110/18	0.91/7.9	470/74	0.91/7.9
25	DHP5α	0.2	2(9%,17%)	---	---	---	---	---	---	390/61	1.3/10	1100/170	0.8/8.2
26	THP3α5α	0.02	0.5(1.9%,11%)	0.43/0.068	13/15	1.5/0.24	11/12	5.5/0.87	4.5/13	200/32	1.6/8.2	150/24	1/8.1
27	THP3β5α	0.02	0.5(4%,15%)	1.6/0.25	3.5/9.8	3.8/0.59	5.1/8.1	5.3/0.83	2.3/11	110/18	1.6/7.6	240/38	3.8/7.8

Table 3., continued.

ID	Steroid	LOD [pg]	LOQ [pg] (bias + precision at LOQ)	Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
				Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]
28	DHP5β	0.7	8(-12%,4.8%)	---	---	---	---	---	---	20/3.1	14/14	280/45	4.1/6.8
29	THP3α5β	0.04	0.5(-9.9%,10%)	---	---	---	---	---	---	130/20	1.1/7.1	180/29	1.4/7.4
30	THP3β5β	0.03	0.1(14%,6.9%)	---	---	---	---	---	---	8.9/1.4	1.6/8.6	22/3.4	1/6.7
31	THP5α20α	0.2	0.5(-4.3%,11%)	1.5/0.24	11/7.1	3.8/0.6	5.2/12	8.9/1.4	5.9/13	220/34	0.65/7.1	390/62	1.1/5.7
32	PD3α5α20α	0.6	0.5(16%,6.1%)	1.7/0.26	5.3/5	2.6/0.41	4.9/12	7/1.1	8.5/12	160/25	2.2/7.5	63/9.8	4.7/7.2
33	PD3β5α20α	2	0.5(17%,3.5%)	9/1.4	12/12	15/2.4	14/8.8	23/3.6	8.7/10	470/73	4.1/7.7	580/90	2.4/7.2
34	THP5β20α	0.2	0.5(1.9%,10%)	---	---	---	---	---	---	15/2.3	2.1/10	250/40	1.3/4.6
35	PD3α5β20α	0.2	0.5(-2.2%,16%)	1.8/0.28	14/11	1.5/0.23	9.2/15	2.2/0.35	14/9.3	52/8.2	2/8.1	70/11	1.4/8.7
36	PD3β5β20α	0.5	0.5(6.4%,13%)	---	---	---	---	---	---	5.4/0.85	13/14	15/2.3	9.8/8.4
37	PD3α5α17	0.2	0.1(-1.8%,13%)	0.51/0.077	19/14	0.44/0.066	11/11	0.42/0.063	12/13	4.6/0.69	6.7/10	6/0.9	4.9/11
38	PD3α5β17	0.1	0.5(-3.5%,13%)	0.8/0.12	13/9.1	0.48/0.072	8.6/14	1.2/0.18	9.7/11	9.4/1.4	3.2/8.2	11/1.7	1.8/5.8
39	PT3α5α17α20α	0.07	0.5(0.56%,11%)	1.9/0.28	3/14	1.3/0.2	3.9/7.9	1.5/0.22	5.6/5.1	1.5/0.23	4.2/15	0.87/0.13	5.1/15
40	PT3β5α17α20α	0.1	0.5(6%,9.4%)	1.6/0.24	3/8.5	2.1/0.31	1.9/8.8	2.2/0.33	2.1/5.5	1.6/0.24	2.5/14	0.81/0.12	5.3/15
41	PT3α5β17α20α	0.06	0.5(0.33%,12%)	10/1.5	1.9/9.1	10/1.5	2.5/8.5	11/1.7	1.2/3.4	49/7.3	1/12	20/3	0.65/11
42	DHA5α	0.3	0.5(3.6%,12%)	1.6/0.27	5.7/8.8	1.8/0.32	13/9	1.4/0.24	11/13	2.8/0.49	8.1/4.9	3.5/0.6	8.6/9.2
43	THA3α5α	0.1	0.5(5.7%,7.5%)	2.7/0.46	2.6/12	1.8/0.31	1.7/11	1.7/0.29	4.8/12	4.1/0.7	4.6/8.6	2.3/0.4	6.7/6.9
44	THA3β5α	0.03	0.5(9.9%,6.6%)	1.8/0.31	1.9/12	2.3/0.4	1.1/8.9	1.9/0.33	3/8	3.3/0.57	3.3/8.8	1.8/0.31	2.1/13
45	THA3α5β	0.01	0.5(5.6%,6.2%)	1.5/0.25	2.8/6.2	1.8/0.31	4.2/6.9	1.4/0.24	3.2/5.6	3.1/0.54	4.7/14	3.7/0.63	6.5/6.5
46	AD3α5α17β	0.2	0.5(5.3%,11%)	1.7/0.29	4.1/9.7	0.49/0.084	8.5/9.2	0.46/0.078	7.1/6.7	0.76/0.13	9.2/13	---	6.7/11
47	AD3β5α17β	0.02	0.5(16%,7.8%)	0.81/0.14	9.4/11	0.7/0.12	12/10	0.58/0.1	10/7.9	0.64/0.11	8.8/11	---	6.4/12
48	AD3α5β17β	0.09	0.5(0.74%,6.5%)	0.93/0.16	8.4/11	0.64/0.11	8.4/9.4	0.93/0.16	13/5.5	0.55/0.095	5.1/15	---	13/15
49	F	30	100(-4.7%,8.8%)	2200/310	2.9/11	2200/300	5.9/5.8	2200/310	3.9/5.6	4900/680	2.9/6.2	1900/260	2.8/4.7
50	E	30	100(-0.35%,6.6%)	370/51	4.6/8.2	350/49	9/10	360/50	7.4/9.8	1000/140	4/6.1	2200/310	5.4/7.1
51	B	1	2(3.2%,10%)	90/13	4.7/9.8	97/14	2.2/7.1	76/11	4.1/7.7	510/74	1.3/6.3	120/18	2.9/8.9
52	DOF	0.8	0.5(1.3%,12%)	1.3/0.19	11/13	1.5/0.22	11/7.4	1.7/0.24	7.6/8.5	5.2/0.75	13/10	5.3/0.76	11/8.4
53	DOC	2	8(-4.7%,11%)	---	---	---	---	---	---	23/3.3	7.3/10	49/7.1	12/6.7
54	THB3α5α	0.5	0.5(-4.9%,3.4%)	1.9/0.27	9.9/11	2.6/0.37	14/9.2	1.9/0.27	11/14	2.6/0.37	12/10	---	---

Table 3., continued.

ID	Steroid	LOD [pg]	LOQ [pg] (bias + precision at LOQ)	Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
				Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]
55	THB3α5β	0.4	0.5(6.6%,17%)	3.5/0.5	15/12	3.6/0.51	14/9	3.3/0.47	13/12	3.4/0.48	12/11	1.1/0.16	2.9/15
56	11OHA4	0.9	8(-3.8%,11%)	470/77	3.4/6.5	450/75	1.9/14	280/47	3.5/15	1600/270	1.9/9.1	600/100	1.4/9.9
57	THA3α5α11β	0.04	0.5(3.3%,10%)	50/8.2	2.4/11	23/3.7	2/6.8	23/3.7	3.6/13	7.3/1.2	4.3/13	8/1.3	5.1/8.2
58	THA3β5α11β	0.04	0.5(6.5%,6.7%)	3/0.49	8.2/11	1.6/0.26	9.3/12	1.6/0.26	9.8/13	0.61/0.1	15/14	1/0.17	9.3/14
59	THA3α5β11β	0.05	0.5(7.7%,6%)	32/5.3	2.9/12	30/4.9	2/13	23/3.8	2.6/13	18/3	2.7/15	32/5.2	2.4/9.3
60	PregC	5	30(10%,2.5%)	1600/250	1.3/7.2	1600/250	1.3/12	1900/300	0.75/9.5	3300/530	1.7/11	28000/4400	0.86/13
61	Preg17C	1	8(1.5%,6.2%)	270/41	1.3/10	280/42	1.6/11	390/59	2.9/9.8	730/110	1.3/7.3	39000/5800	0.93/8.6
62	DHPreg20αC	4	30(3.2%,8.2%)	8900/1400	0.76/11	6300/990	0.66/14	9500/1500	1.1/4.9	5000/790	1.3/9.1	15000/2300	0.91/7.8
63	DHEAC	0.2	30(-0.17%,4.3%)	27000/4700	1.3/7.3	26000/4600	1.2/6	25000/4400	1.3/4.1	12000/2100	0.59/5.5	29000/5000	0.51/6.6
64	5-AdiolC	4	30(5.9%,9.2%)	20000/3400	1.2/13	17000/2900	0.78/12	13000/2300	0.54/13	2100/360	1.2/11	26000/4500	1.3/7.7
65	AT16αC	0.7	8(-0.063%,9.4%)	310/50	2.8/12	410/67	1.2/11	370/61	1.2/13	1000/170	1.3/12	14000/2300	0.37/11
66	DHP17α20αC	5	8(5.2%,7.2%)	86/13	1.5/14	130/20	7.2/11	140/21	4.8/13	---	---	---	---
67	DHP20αC	2	8(1.9%,12%)	18/2.9	2.8/9	23/3.6	4/10	52/8.2	4.9/12	190/30	1.5/13	880/140	1.5/8.2
68	TC	2	8(8.2%,8%)	---	---	---	---	---	---	110/19	5.3/12	290/51	3.7/10
69	EpiTC	5	8(6.4%,10%)	---	---	---	---	---	---	92/16	5.8/9.3	2400/410	0.76/4.2
70	E1C	10	8(3.2%,7.9%)	---	---	---	---	---	---	3700/680	9.4/9	200/37	0.42/3.4
71	E2C	0.2	2(3.5%,8.7%)	---	---	---	---	---	---	160/29	1.1/14	29/5.3	7.2/10
72	E3C	0.6	8(3.9%,9.4%)	---	---	---	---	---	---	3300/530	1.7/11	28000/4400	0.86/13
73	THP3α5αC	0.3	2(-2.8%,4.1%)	46/7.3	3/14	89/14	3.1/14	430/68	1.6/5.8	9500/1500	0.97/7.2	2600/410	0.75/8.2
74	THP3β5αC	0.3	8(4%,9%)	110/17	3.2/9.6	160/25	1.8/12	290/45	1.8/11	6400/1000	1/9.6	3100/490	0.89/13
75	THP3α5βC	0.7	2(0.29%,5.5%)	200/31	1.9/9.9	260/41	2.7/12	450/70	0.9/10	5600/880	0.99/7.7	2900/450	1/7
76	THP3β5βC	0.5	2(-5.5%,5.4%)	25/3.9	2.9/11	76/12	3/8.5	89/14	1.5/15	1500/240	0.94/7.3	1000/160	0.73/4.7
77	THP5α20αC	5	2(2.3%,8.7%)	4/0.63	8.3/6.9	8.9/1.4	6.6/15	89/14	5.5/11	830/130	1.7/13	1100/180	1.1/15
78	PD3α5α20αC	10	8(1.4%,8.8%)	210/33	2.1/14	400/62	3.1/7.2	1700/270	0.58/12	33000/5100	1/10	19000/3000	0.35/11
79	PD3β5α20αC	5	30(-7.3%,7.2%)	3200/500	5.2/8.9	17000/2700	12/9.4	50000/7800	12/12	420000/65000	0.96/8.9	220000/35000	0.47/8.8
80	THP5β20αC	6	30(-9.8%,18%)	---	---	---	---	---	---	220/35	1.1/13	620/98	0.47/12
81	PD3α5β20αC	2	8(7.4%,6.2%)	130/20	2.5/11	330/52	1.4/9.6	1400/220	1.2/13	11000/1700	0.73/11	13000/2000	0.83/8.6

Table 3., continued.

ID	Steroid	LOD [pg]	LOQ [pg] (bias + precision at LOQ)	Men		Women, follicular phase		Women, luteal phase		Women, pregnancy		Mixed umbilical blood	
				Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]	Level [pg inj.]/ [nM]	Intra-/Inter-assay [%]
82	PD3β5β20αC	9	8(4.9%,7.7%)	150/24	5.6/12	770/120	2.9/11	1200/190	1.4/14	7700/1200	0.98/9.7	6300/990	0.51/13
83	PD3α5α17C	0.5	2(-1.6%,7.6%)	29/4.4	4.8/7.5	15/2.2	5.7/6.9	41/6.1	3.5/6.7	150/22	0.88/7.6	63/9.5	2.6/7.8
84	PD3α5β17C	0.3	8(-3.2%,10%)	120/18	1.5/6.7	80/12	3.4/7.8	170/26	0.89/9.1	670/100	0.32/6.9	460/69	1.4/6.8
85	PT3α5α17α20αC	0.2	8(0.31%,6%)	---	---	---	---	---	---	260/39	4.4/14	550/82	14/11
86	PT3β5α17α20αC	0.2	8(-5.8%,17%)	---	---	---	---	---	---	52/7.7	4.9/14	35/5.2	2.9/12
87	PT3α5β17α20αC	0.2	8(1.3%,13%)	---	---	---	---	---	---	2400/360	1.8/13	2600/390	4.1/15
88	THA3α5αC	1	8(2%,6.8%)	11000/1900	0.67/8.6	16000/2700	2.1/11	19000/3200	0.77/13	3700/630	0.68/7.9	580/100	0.91/14
89	THA3β5αC	0.9	8(-0.81%,8.5%)	3000/510	1.2/7.6	3000/510	1.4/11	2900/500	1.4/5.7	870/150	1.2/5.9	320/55	0.73/6.9
90	THA3α5βC	3	8(-2.5%,20%)	580/100	0.63/5	750/130	0.7/5.9	640/110	0.67/8.2	360/62	0.81/7	120/20	1.2/7.6
91	THA3β5βC	0.9	8(-1.6%,8.2%)	310/54	0.97/8.7	420/73	2.3/12	570/99	0.94/14	58/10	2.2/10	15/2.5	3.8/10
92	AD3α5α17βC	1	8(-0.64%,5.8%)	990/170	0.98/10	460/78	1.9/6.5	440/76	0.94/13	180/31	0.82/8.6	190/33	0.99/11
93	AD3β5α17βC	0.1	8(-1.6%,6%)	1500/250	1.2/8.6	870/150	1.8/11	1200/200	5.9/12	170/29	1.8/8.4	87/15	3.7/8.1
94	AD3α5β17βC	2	8(2.2%,11%)	140/24	2.5/11	130/22	1.9/12	140/24	3.1/12	33/5.6	2.8/12	52/8.9	4.7/12
95	AD3β5β17βC	2	2(11%,13%)	7/1.2	4.4/11	7/1.2	12/13	12/2.1	4.6/12	4/0.69	12/14	9.3/1.6	9.2/11
96	THB3α5αC	20	30(3.1%,12%)	---	---	---	---	690/98	9.4/10	280/40	7/14	140/20	5.3/14
97	THB3α5βC	10	30(3.5%,11%)	---	---	---	---	---	---	330/47	7.9/14	---	---
98	THA3α5α11βC	0.2	2(8.6%,9%)	270/44	0.98/5.1	230/38	0.91/7.8	230/37	1.3/8.2	120/19	1.7/7.7	86/14	2.4/5.1
99	THA3β5α11βC	0.2	2(-8.1%,8.6%)	13/2.2	6.3/6.6	14/2.3	7.5/7.6	12/2	7.2/9.9	6.7/1.1	2.8/14	61/10	2.9/6.8
100	THA3α5β11βC	0.3	2(6.2%,7.7%)	56/9.2	1.2/11	92/15	2/13	92/15	1.8/13	20/3.2	3.8/5.5	6.7/1.1	3.8/12

Limit of detection and limit of quantification

The lowest nonzero standard on the calibration curve defined the sensitivity. The analyte response at the LOQ was at least five times the analyte response of the zero calibrator and the bias at the LOQ was at most ±20 % of nominal concentration (as found using replicates prepared in six different runs). Similarly, the imprecision was at most ±20 % RSD as found using six replicates in four runs (Bioanalytical Method Validation 2018) (Table 3).

The LOD was sufficient in all cases where the intra- and/or inter-assay for precision did not exceeded the 15 % RSD (Table 3) but the LOQ was borderline for the levels of AD3α5β17β and AD3α5β17β, E1 and PD3α5β17 levels in subjects out of pregnancy, and E2 and THP3α5α levels in men.

Specificity/selectivity of the method

In the co-eluting steroids, the selectivity was tested by injecting large amounts of the individual steroids and checking the potential contribution to other steroids respecting circulating levels of the potential interferents. For instance, for the transition 360>84 between DHEA and epiandrosterone we found some interference. On the other hand, the interference of DHEA for transition 360>270 in epiandrosterone was absent. Therefore, we choose the transition for quantitation of epiandrosterone 360>270 instead of the 360>84 transition. We also tested partly co-eluting pregnenolone and isopregnanolone and found some interference on 388>70 but no interference on 388>173 transition, which was then chosen for quantitation of isopregnanolone. The interferences were also tested for 388>70 transition between partly co-eluting 7β-OH-DHEA and allopregnanolone but there was no perceptible interference. Some interference was found for 421>255 transition between partly co-eluting 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol but the corresponding peaks only marginally coincided and the quantitation was possible. Besides the cases mentioned above and DOF, Preg16α, T16α, in which only a single MRM transition was recorded, no further perceptible interferences were found and the remaining ion ratios were within the tolerance according to WADA Technical Document – TD2010IDCR "Identification Criteria for Qualitative Assays Incorporating Column Chromatography and Mass Spectrometry".

The levels of DOF were higher in male serum pool when compared with our previously published data from RIA assays (Hill *et al.* 1995), possibly due to the unintentional inclusion of patients with Cushing syndrome or congenital adrenal hyperplasia in some pooled samples. However, the recording of a single MRM transition for DOF did not rule out the possibility of some endogenous co-eluting interference being responsible of the apparent larger concentrations.

Efficiency of methanolysis and stability of non-deuterated and deuterated steroids

The deconjugating step in the present method was performed using the methanolysis according to Dehennin *et al.* (1996). This harsh acid hydrolysis is an adopted method of deconjugation that efficiently and rapidly cleaves both sulfates and glucuronides simultaneously. However, the formation of artefactual by-products is a known weakness of this method (Dehennin *et al.* 1996, Shackleton *et al.* 2004, Viljanto *et al.* 2018).

The results characterizing the efficiency of methanolysis for seven steroids sulfates/disulfates are summarized in Table S5. The efficiency of the methanolysis step for individual steroid sulfates was high, ranging from 85 % to 116 % (98±11 %, shown as mean ± SD) (Table S5). Furthermore, we have tested the methanolysis efficiency for the DHEA sulfate using the same protocol but sulfated D6-DHEA as the internal standard. The efficiency of methanolysis step was close to absolute and almost the same when using the unconjugated or sulfated D6-DHEA as the internal standard (102.6±0.9 %, shown as mean ± SD).

Considering the high efficiency of the methanolysis step, there is probably lessened necessity to use sulfated internal standards instead of the unconjugated ones as the deconjugation step does not represent a critical point in methanolysis. Thus, the more available unconjugated deuterated steroids may be used as satisfactory surrogates instead of their more appropriate conjugated equivalents. However, all internal standards (regardless their conjugation status) should possess isotopic stability in strongly acidic environment, which occurs during the methanolysis.

Some steroids have also limited chemical stability during the methanolysis (Dehennin *et al.* 1996, Viljanto *et al.* 2018). Dehennin *et al.* (1996) report, that while the sulfates of androsterone, epitestosterone, testosterone, 5-androstene-3β,17β-diol (5-Adiol) and DHEA and glucuronides of androsterone and testosterone are almost totally recovered using the methanolysis,

steroids with tertiary alcohol in the steroid 17 position and secondary alcohol in the steroid 11β-position may dehydrate in strongly acidic conditions. This environment stimulates a protonation of the oxygen attached to steroid C-17 position and the nucleophilic attack by methanol, which consequently induces a cleavage of sulfate and glucuronide moieties on steroid molecules. However, there is also a risk of partial dehydration and formation of double bond (Viljanto *et al.* 2018).

The analysis of steroid chemical stability of unconjugated steroids (see section *Efficiency of methanolysis and stability of non-deuterated and deuterated steroids*) showed that most of them were relatively stable during the methanolysis. However, estrogens, 16α-hydroxy-metabolite of 5-Adiol and 11β-hydroxy- and 3-oxo- steroids showed a limited stability (Table S6). Nevertheless, even in these cases, one can expect a similar degree of conversion to artefacts in standard and unknown samples on condition that they are processed in the same way in one run. So, the obtained results may be still acceptable as apparent in tables presenting analytical criteria for conjugated forms of steroids (see section ***Validation parameters***). We are aware that the use of chemically and isotopically stable deuterated external standards with sufficient isotopic purity in conjugated forms would be a by far better approach.

The accessibility of appropriate deuterated conjugated internal standards is even more critical. Moreover, the deuterated internal standards are often isotopically unstable. The strongly acidic environment during the methanolysis promotes deuterium-hydrogen exchange, which considerably limits the number of applicable deuterated standards. For instance, a complete deuterium-hydrogen exchange was observed in a deuterium-labelled, D9-progesterone during methanolysis but no change was observed when the samples spiked with D9-progesterone were incubated with methanol in the neutral environment. The deuterium-hydrogen exchange is induced by acid-catalyzed enol tautomer formation when the double bond rapidly moves between the keto and enol forms. Although the equilibrium usually favors the keto-tautomer, it can be shifted to the enol-one by acidic or alkaline environment. Steroids labelled on an α-carbon adjacent to a ketone functional group(s) exhibit the hydrogen exchange, whereas other labelled analytes are unlikely to cause any problems. In extreme situations, such as in the case of D9-progesterone, the deuterium-hydrogen exchange *via* keto-enol tautomerism may lead to the formation of unlabeled product (Viljanto *et al.* 2018). We observed this effect during the methanolysis when using D9-progesterone and D8-17α-hydroxyprogesterone as internal standards. Besides the problems with the isotopic stability, the relatively frequent drawback of deuterated internal standards may be also their insufficient isotopic purity, which is specifically critical in analytes showing wide biological variability such as pregnane steroids exhibiting extreme changes during the menstrual cycle and pregnancy.

In contrast to some authors discriminating between glucuronide, monosulfate and disulfate moieties on steroid molecules, we did not test their levels separately (Mareck *et al.* 2008, Meng *et al.* 1997) but measured only the total polar conjugates. On one hand, the concurrent deconjugation of sulfates and glucuronides is a weakness of our method but on the other hand, the methanolysis is more robust and less laborious then the enzymatic hydrolysis or microcolumn pre-separation of sulfate, disulfate or glucuronide moieties from each other. Nevertheless, the discrimination between these moieties may be desirable in the diagnostics of some disorders such as the intrahepatic cholestasis of pregnancy. In this pathology, from a variety of pregnanediols, only the of 5α-pregnane-3α,20α-diol disulfate is considered as toxic for fetus (Abu-Hayyeh *et al.* 2013, Meng *et al.* 1997).

Comparison of the present GC-MS/MS method with our previous GC-MS method

Due to the high number of analytes and variety of steroids measured in human circulation, a comparison of all steroids with results from other methods was unachievable. Nevertheless, a number of our present results are comparable with those data from our previously published GC-MS method (Bicikova *et al.* 2013, Duskova *et al.* 2012, Hill *et al.* 2010b, Hill *et al.* 2011a, Hill *et al.* 2014, Hill *et al.* 2011b, Hill *et al.* 2010c, Kancheva *et al.* 2011, Majewska *et al.* 2014, Parizek *et al.* 2016, Paskova *et al.* 2014, Pospisilova *et al.* 2012, Vankova *et al.* 2016) as well as with the results of other authors (for review see Hill *et al.* 2010b).

The agreement between GC-MS, LC-MS/MS, RIA (for cortisol) and our present method for individual analytes mostly ranged from satisfactory to excellent results (Table S1 and Table S2, Fig. S1 and Fig. S2) even if there were little deviations from identity line and problems with LDR in two analytes. We compared responses to samples injected in high (2 µl) and low

(0.2 µl) injection volumes for analytes with high circulating levels (some conjugated steroids) and found two of them, in which a considerable number of responses was not proportional to the injected volume (DHEA sulfate and androsterone sulfate). In these analytes, the samples from subjects with lower analyte circulating levels showed tight correlations between concentrations calculated from low and high injection volume and slopes (using the same calibration curve) of the corresponding regression lines did not significantly differ from 1. However, in the samples from subjects with higher analyte concentrations, the divergence between concentrations calculated for samples injected at high and low injection volume began to grow. Here the samples injected in low volume showed higher concentrations when compared with the same ones injected in the high volume (Fig. S3). It is evident that samples from subjects with higher analyte circulating levels underwent the same treatment as those from subject with the lower analyte circulating levels. Thus, the only cause of the differences in the former group should be the different injection volumes. As expected, the only change at lower injection volume was the shift of the analyte response to LDR in the samples from subjects with higher analyte circulating levels without significant influence on results in the samples from subject with the lower analyte circulating levels. These levels evidently remained sufficiently high for analysis at lower injection volumes. Based on these data, the sulfates of DHEA and androsterone were measured at low injection volumes of samples in the present method and the method validation for these steroid conjugated was also completed at low injection volumes.

In addition to the steroids quantified in the previous method (Hill *et al.* 2010b), the present one was extended for corticoids, 11β-hydroxy-androstanes and 17α-hydroxylated 5α/β-reduced pregnanes. The last-mentioned substances may be useful for the investigation of the alternative "backdoor" pathway. When comparing the analytical characteristics of the present and previous methods, the first exhibited by far higher selectivity, generally higher sensitivity and better precision particularly for 17α-hydroxysteroids. However, in the case of estrogens the precision was worse and even unsatisfactory for estrone in non-pregnant subjects, which may be associated with the use of different derivatization agent in the silylation step and worse repeatability (during the drying of derivatized mixture under nitrogen because to its higher heterogeneity in comparison with our previous method). On the other hand, the more intense and lengthier derivatization together with the use of more advanced GC-MS/MS platform resulted in substantially improved sensitivity and precision in 17α-hydroxy-steroids and enabled the quantification of corticoids and 11β-hydroxy-androgens, which were undetectable by our previous method.

Limitations of our method

We acknowledge that our proposed method has some limitations. The first is the absence of conjugated external and deuterated internal standards in most conjugated steroids and absence of deuterated internal standards even for most unconjugated steroids. The first reason was a limited accessibility of these substances. The further serious problem especially in quantification of conjugated steroids was chemical and isotopic instability as well as isotopic impurity of various deuterated standards (as discussed above). Therefore, we excluded the analysis of four steroid conjugates, which were well detectable but extremely instable during the hydrolysis such as conjugated 7α/β-hydroxy-metabolites of DHEA and 5-Adiol. The difficult accessibility, isotopic and chemical instability were also the reasons for which we used only a single (but pure and stable) deuterated steroid conjugate (D6-DHEA sulfate) as the internal standard for the quantification of conjugated steroids.

Furthermore, in spite of wide spectrum of the measured steroids some diagnostically important steroids remained, which were not included. Partly due to unfavorable fragmentation pattern of the steroid even after derivatization resulting in low sensitivity as in the case of 11-deoxycortisol. In addition, 11-deoxycorticosterone was below the LOQ for non-pregnant subjects and 21-deoxycortisol was above the LOQ for all groups but the sensitivity was also relatively low. Also, the sensitivity for estrogens in non-pregnant subjects was low. The quantification of interesting steroids such as 11β-hydroxy-testosterone, 11-oxo-testosterone and 11-oxo-androstenedione was not tested as well as the measurement of steroid 6α/β-hydroxy-catabolites.

Conclusions

To the best of our knowledge, in spite of the limitations described above, this is the first GC-MS/MS method for multicomponent quantitation of circulating

steroids validated for different physiological conditions in humans including gender differences and pregnancy status. In addition, this method currently includes the largest spectrum of human circulating steroids and steroid polar conjugates, at least for the GC-MS/MS platform. As have been demonstrated in our previous papers, steroid profiling enables various pathologies to be rapidly diagnosed (Bicikova *et al.* 2013, Hill *et al.* 2010c, Kanceva *et al.* 2015, Parizek *et al.* 2016, Sosvorova *et al.* 2015, Vankova *et al.* 2016). The present GC-MS/MS method includes a wide range of analytes, which reflect activities of most steroidogenic enzymes. Thus, it could be used for the estimation of changes in steroidogenesis for various physiological and pathophysiological situations and subsequently the data obtained can be utilized for uncovering the mechanisms of some steroid-related human pathologies (Parizek *et al.* 2016, Sterzl *et al.* 2017, Vankova *et al.* 2016).

Nevertheless, the hydrolysis step is laborious and may carry problems with stability of some steroid conjugates. Furthermore, some positions of sulfate or glucuronide groups in steroid molecule may be resistant to hydrolysis although the deconjugation step used in the present method appears to be quite efficient. Moreover, the physiological and pathophysiological importance of steroid sulfates and glucuronides may be different. Therefore, the future work in steroid assay development should strive to measure the entire conjugated molecule without hydrolysis.

Conflict of Interest

There is no conflict of interest.

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References

ABU-HAYYEH S, PAPACLEOVOULOU G, LOVGREN-SANDBLOM A, TAHIR M, ODUWOLE O, JAMALUDIN NA, RAVAT S, NIKOLOVA V, CHAMBERS J, SELDEN C, REES M, MARSCHALL HU, PARKER MG, WILLIAMSON C: Intrahepatic cholestasis of pregnancy levels of sulfated progesterone metabolites inhibit farnesoid X receptor resulting in a cholestatic phenotype. *Hepatology* **57**: 716-726, 2013.

ANDRASI N, MOLNAR B, DOBOS B, VASANITS-ZSIGRAI A, ZARAY G, MOLNAR-PERL I: Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube River samples by gas chromatography, tandem mass spectrometry. *Talanta* **115**: 367-373, 2013.

BICIKOVA M, HILL M, RIPOVA D, MOHR P, HAMPL R: Determination of steroid metabolome as a possible tool for laboratory diagnosis of schizophrenia. *J Steroid Biochem Mol Biol* **133**: 77-83, 2013.

BIOANALYTICAL METHOD VALIDATION. Guidance for Industry. BIOPHARMACEUTICS (ed.), 2018.

BLAND JM, ALTMAN DG: Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1**: 307-310, 1986.

BLOKLAND MH, VAN TRICHT EF, VAN ROSSUM HJ, STERK SS, NIELEN MW: Endogenous steroid profiling by gas chromatography-tandem mass spectrometry and multivariate statistics for the detection of natural hormone abuse in cattle. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **29**: 1030-1045, 2012.

BROCHU M, BELANGER A: Comparative study of plasma steroid and steroid glucuronide levels in normal men and in men with benign prostatic hyperplasia. *Prostate* **11**: 33-40, 1987.

BROCHU M, BELANGER A, DUPONT A, CUSAN L, LABRIE F: Effects of flutamide and aminoglutethimide on plasma 5 alpha-reduced steroid glucuronide concentrations in castrated patients with cancer of the prostate. *J Steroid Biochem* **28**: 619-622, 1987.

CHOI MH, CHUNG BC: Diagnostic evaluation of enzyme activity related to steroid metabolism by mass spectrometry-based steroid profiling. *Mass Spectrometry Letters* **5**: 35-41, 2014.

CHRISTAKOUDI S, COWAN DA, TAYLOR NF: Steroids excreted in urine by neonates with 21-hydroxylase deficiency: characterization, using GC-MS and GC-MS/MS, of the D-ring and side chain structure of pregnanes and pregnenes. *Steroids* **75**: 34-52, 2010.

CHRISTAKOUDI S, COWAN DA, TAYLOR NF: Steroids excreted in urine by neonates with 21-hydroxylase deficiency. 2. Characterization, using GC-MS and GC-MS/MS, of pregnanes and pregnenes with an oxo- group on the A- or B-ring. *Steroids* **77**: 382-393, 2012a.

CHRISTAKOUDI S, COWAN DA, TAYLOR NF: Steroids excreted in urine by neonates with 21-hydroxylase deficiency. 3. Characterization, using GC-MS and GC-MS/MS, of androstanes and androstenes. *Steroids* **77**: 1487-1501, 2012b.

CHRISTAKOUDI S, COWAN DA, TAYLOR NF: Steroids excreted in urine by neonates with 21-hydroxylase deficiency. 4. Characterization, using GC-MS and GC-MS/MS, of 11oxo-pregnanes and 11oxo-pregnenes. *Steroids* **78**: 468-475, 2013.

COURANT F, AKSGLAEDE L, ANTIGNAC JP, MONTEAU F, SORENSEN K, ANDERSSON AM, SKAKKEBAEK NE, JUUL A, BIZEC BL: Assessment of circulating sex steroid levels in prepubertal and pubertal boys and girls by a novel ultrasensitive gas chromatography-tandem mass spectrometry method. *J Clin Endocrinol Metab* **95**: 82-92, 2010.

DEHENNIN L, PERES G: Plasma and urinary markers of oral testosterone misuse by healthy men in presence of masking epitestosterone administration. *Int J Sports Med* **17**: 315-319, 1996.

DEHENNIN L, LAFARGE P, DAILLY P, BAILLOUX D, LAFARGE JP: Combined profile of androgen glucuro- and sulfoconjugates in post-competition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry. *J Chromatogr B Biomed Appl* **687**: 85-91, 1996.

DUSKOVA M, SIMUNKOVA K, HILL M, VELIKOVA M, KUBATOVA J, KANCHEVA L, KAZIHNITKOVA H, HRUSKOVICOVA H, POSPISILOVA H, RACZ B, SALATOVA M, CIRMANOVA V, KRALIKOVA E, STARKA L, PARIZEK A: Chronic cigarette smoking alters circulating sex hormones and neuroactive steroids in premenopausal women. *Physiol Res* **61**: 97-111, 2012.

ESQUIVEL A, POZO OJ, GARROSTAS L, BALCELLS G, GOMEZ C, KOTRONOULAS A, JOGLAR J, VENTURA R: LC-MS/MS detection of unaltered glucuronoconjugated metabolites of metandienone. *Drug Test Anal* **9**: 534-544, 2017.

GAMBELUNGHE C, SOMMAVILLA M, FERRANTI C, ROSSI R, ARONI K, MANES N, BACCI M: Analysis of anabolic steroids in hair by GC/MS/MS. *Biomed Chromatogr* **21**: 369-375, 2007.

GOMES RL, MEREDITH W, SNAPE CE, SEPHTON MA: Conjugated steroids: analytical approaches and applications. *Anal Bioanal Chem* **393**: 453-458, 2009.

HA YW, MOON JY, JUNG HJ, CHUNG BC, CHOI MH: Evaluation of plasma enzyme activities using gas chromatography-mass spectrometry based steroid signatures. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**: 4125-4132, 2009.

HANEEF J, SHAHARYAR M, HUSAIN A, RASHID M, MISHRA R, PARVEEN S, AHMED N, PAL M, KUMAR D: Application of LC-MS/MS for quantitative analysis of glucocorticoids and stimulants in biological fluids. *J Pharm Anal* **3**: 341-348, 2013.

HANSEN M, JACOBSEN NW, NIELSEN FK, BJORKLUND E, STYRISHAVE B, HALLING-SORENSEN B: Determination of steroid hormones in blood by GC-MS/MS. *Anal Bioanal Chem* **400**: 3409-3417, 2011.

HILL M, LAPCIK O, HAMPL R, STARKA L, PUTZ Z: Radioimmunoassay of three deoxycorticoids in human plasma following HPLC separation. *Steroids* **60**: 615-620, 1995.

HILL M, PARIZEK A, CIBULA D, KANCHEVA R, JIRASEK JE, JIRKOVSKA M, VELIKOVA M, KUBATOVA J, KLIMKOVA M, PASKOVA A, ZIZKA Z, KANCHEVA L, KAZIHNITKOVA H, ZAMRAZILOVA L, STARKA L: Steroid metabolome in fetal and maternal body fluids in human late pregnancy. *J Steroid Biochem Mol Biol* **122**: 114-132, 2010a.

HILL M, PARIZEK A, KANCHEVA R, DUSKOVA M, VELIKOVA M, KRIZ L, KLIMKOVA M, PASKOVA A, ZIZKA Z, MATUCHA P, MELOUN M, STARKA L: Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and in amniotic fluid in normal and preterm labor. *J Steroid Biochem Mol Biol* **121**: 594-610, 2010b.

HILL M, ZARUBOVA J, MARUSIC P, VRBIKOVA J, VELIKOVA M, KANCHEVA R, KANCHEVA L, KUBATOVA J, DUSKOVA M, ZAMRAZILOVA L, KAZIHNITKOVA H, SIMUNKOVA K, STARKA L: Effects of valproate and carbamazepine monotherapy on neuroactive steroids, their precursors and metabolites in adult men with epilepsy. *J Steroid Biochem Mol Biol* **122**: 239-252, 2010c.

HILL M, PARIZEK A, VELIKOVA M, KUBATOVA J, KANCHEVA R, DUSKOVA M, SIMUNKOVA K, KLIMKOVA M, PASKOVA A, ZIZKA Z, JIRASEK JE, JIRKOVSKA M, STARKA L: The distribution of placental oxidoreductase isoforms provides different milieus of steroids influencing pregnancy in the maternal and fetal compartment. *Horm Mol Biol Clin Invest* **4**: 581-600, 2011a.

HILL M, VRBIKOVA J, ZARUBOVA J, KANCHEVA R, VELIKOVA M, KANCHEVA L, KUBATOVA J, DUSKOVA M, MARUSIC P, PARIZEK A, STARKA L: The steroid metabolome in lamotrigine-treated women with epilepsy. *Steroids* **76**: 1351-1357, 2011b.

HILL M, PASKOVA A, KANCEVA R, VELIKOVA M, KUBATOVA J, KANCHEVA L, ADAMCOVA K, MIKESOVA M, ZIZKA Z, KOUCKY M, SARAPATKOVA H, KACER V, MATUCHA P, MELOUN M, PARIZEK A: Steroid profiling in pregnancy: a focus on the human fetus. *J Steroid Biochem Mol Biol* **139**: 201-222, 2014.

IMPENS S, VAN LOCO J, DEGROODT JM, DE BRABANDER H: A downscaled multi-residue strategy for detection of anabolic steroids in bovine urine using gas chromatography tandem mass spectrometry (GC-MS3). *Anal Chim Acta* **586**: 43-48, 2007.

KANCEVA R, STARKA L, KANCHEVA L, HILL M, VELIKOVA M, HAVRDOVA E: Increased serum levels of C21 steroids in female patients with multiple sclerosis. *Physiol Res* **64** (Suppl 2): S247-S254, 2015.

KANCHEVA R, HILL M, NOVAK Z, CHRASTINA J, KANCHEVA L, STARKA L: Neuroactive steroids in periphery and cerebrospinal fluid. *Neuroscience* **191**: 22-27, 2011.

KELLY C: Analysis of steroids in environmental water samples using solid-phase extraction and ion-trap gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. *J Chromatogr A* **872**: 309-314, 2000.

KIM B, MOON JY, CHOI MH, YANG HH, LEE S, LIM KS, YOON SH, YU KS, JANG IJ, CHO JY: Global metabolomics and targeted steroid profiling reveal that rifampin, a strong human PXR activator, alters endogenous urinary steroid markers. *J Proteome Res* **12**: 1359-1368, 2013.

KRONE N, HUGHES BA, LAVERY GG, STEWART PM, ARLT W, SHACKLETON CH: Gas chromatography/ mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). *J Steroid Biochem Mol Biol* **121**: 496-504, 2010.

KURECKOVA K, MARALIKOVA B, VENTURA K: Supercritical fluid extraction of steroids from biological samples and first experience with solid-phase microextraction-liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* **770**: 83-89, 2002.

LABRIE F, BELANGER A, CUSAN L, GOMEZ JL, CANDAS B: Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J Clin Endocrinol Metab* **82**: 2396-2402, 1997.

MAJEWSKA MD, HILL M, URBANOWICZ E, ROK-BUJKO P, BIENKOWSKI P, NAMYSLOWSKA I, MIERZEJEWSKI P: Marked elevation of adrenal steroids, especially androgens, in saliva of prepubertal autistic children. *Eur Child Adolesc Psychiatry* **23**: 485-498, 2014.

MANUILOVA E, SCHUETZENMEISTER A, MODEL F: *Method Comparison Regression*. https://CRAN.R-project.org/package=mcr, 2014.

MARCOS J, PASCUAL JA, DE LA TORRE X, SEGURA J: Fast screening of anabolic steroids and other banned doping substances in human urine by gas chromatography/tandem mass spectrometry. *J Mass Spectrom* **37**: 1059-1073, 2002.

MARCOS J, RENAU N, CASALS G, SEGURA J, VENTURA R, POZO OJ: Investigation of endogenous corticosteroids profiles in human urine based on liquid chromatography tandem mass spectrometry. *Anal Chim Acta* **812**: 92-104, 2014.

MARECK U, GEYER H, OPFERMANN G, THEVIS M, SCHANZER W: Factors influencing the steroid profile in doping control analysis. *J Mass Spectrom* **43**: 877-891, 2008.

MATYSIK S, SCHMITZ G: Determination of steroid hormones in human plasma by GC-triple quadrupole MS. *Steroids* **99**: 151-154, 2015.

MENG LJ, REYES H, AXELSON M, PALMA J, HERNANDEZ I, RIBALTA J, SJOVALL J: Progesterone metabolites and bile acids in serum of patients with intrahepatic cholestasis of pregnancy: effect of ursodeoxycholic acid therapy. *Hepatology* **26**: 1573-1579, 1997.

MOON JY, JUNG HJ, MOON MH, CHUNG BC, CHOI MH: Heat-map visualization of gas chromatography-mass spectrometry based quantitative signatures on steroid metabolism. *J Am Soc Mass Spectrom* **20**: 1626-1637, 2009.

MOON JY, CHOI MH, KIM J: Metabolic profiling of cholesterol and sex steroid hormones to monitor urological diseases. *Endocr Relat Cancer* **23**: R455-R467, 2016.

NILSSON ME, VANDENPUT L, TIVESTEN A, NORLEN AK, LAGERQUIST MK, WINDAHL SH, BORJESSON AE, FARMAN HH, POUTANEN M, BENRICK A, MALIQUEO M, STENER-VICTORIN E, RYBERG H, OHLSSON C: Measurement of a comprehensive sex steroid profile in rodent serum by high-sensitive gas chromatography-tandem mass spectrometry. *Endocrinology* **156**: 2492-2502, 2015.

PARIZEK A, HILL M, DUSKOVA M, VITEK L, VELIKOVA M, KANCHEVA R, SIMJAK P, KOUCKY M, KOKRDOVA Z, ADAMCOVA K, CERNY A, HAJEK Z, STARKA L: A comprehensive evaluation of steroid metabolism in women with intrahepatic cholestasis of pregnancy. *PLoS One* **11**: e0159203, 2016.

PASKOVA A, PARIZEK A, HILL M, VELIKOVA M, KUBATOVA J, DUSKOVA M, ADAMCOVA K, KOUCKY M, SIMJAK P, CERNY A, STARKA L: Steroid metabolome in the umbilical cord: is it necessary to differentiate between arterial and venous blood? *Physiol Res* **63**: 115-126, 2014.

POSPISILOVA H, VANKOVA M, HILL M, MELOUN M, BENDLOVA B, DUSKOVA M, STARKA L: The differences between aromatizable and non-aromatizable androgens in relation to body composition and metabolic syndrome risk factors in men. *J Steroid Biochem Mol Biol* **132**: 105-111, 2012.

RARO M, PORTOLES T, PITARCH E, SANCHO JV, HERNANDEZ F, GARROSTAS L, MARCOS J, VENTURA R, SEGURA J, POZO OJ: Potential of atmospheric pressure chemical ionization source in gas chromatography tandem mass spectrometry for the screening of urinary exogenous androgenic anabolic steroids. *Anal Chim Acta* **906**: 128-138, 2016.

ROSSI SA, JOHNSON JV, YOST RA: Short-column gas chromatography/tandem mass spectrometry for the detection of underivatized anabolic steroids in urine. *Biol Mass Spectrom* **23**: 131-139, 1994.

SANCHEZ-GUIJO A, OJI V, HARTMANN MF, TRAUPE H, WUDY SA: Simultaneous quantification of cholesterol sulfate, androgen sulfates, and progestagen sulfates in human serum by LC-MS/MS. *J Lipid Res* **56**: 1843-1851, 2015.

SHACKLETON C, MARCOS J, ARLT W, HAUFFA BP: Prenatal diagnosis of P450 oxidoreductase deficiency (ORD): a disorder causing low pregnancy estriol, maternal and fetal virilization, and the Antley-Bixler syndrome phenotype. *Am J Med Genet A* **129A**: 105-112, 2004.

SHEN M, XIANG P, SHEN B, WANG M: Determination of endogenous anabolic steroids in hair using gas chromatography-tandem mass spectrometry (in Chinese). *Se Pu* **26**: 454-459, 2008.

SOLDIN SJ, SOLDIN OP: Steroid hormone analysis by tandem mass spectrometry. *Clin Chem* **55**: 1061-1066, 2009.

SOSVOROVA L, HILL M, MOHAPL M, VITKU J, HAMPL R: Steroid hormones in prediction of normal pressure hydrocephalus. *J Steroid Biochem Mol Biol* **152**: 124-132, 2015.

STERZL I, HILL M, STARKA L, VELIKOVA M, KANCEVA R, JEMELKOVA J, CZERNEKOVA L, KOSZTYU P, ZADRAZIL J, MATOUSOVIC K, VONDRAK K, RASKA M: Patients with IgA nephropathy have altered levels of immunomodulatory C19 steroids. Glucocorticoid therapy with addition of adrenal androgens may be the choice. *Physiol Res* **66** (Suppl 3): S433-S442, 2017.

STYRISHAVE B, PEDERSEN KE, CLARKE O, HANSEN M, BJORKLUND E, SONNE C, DIETZ R: Steroid hormones in multiple tissues of East Greenland polar bears (Ursus maritimus). *Polar Biol* **70**: 37-49, 2017.

TOKUSHIGE K, HASHIMOTO E, KODAMA K, TOBARI M, MATSUSHITA N, KOGISO T, TANIAI M, TORII N, SHIRATORI K, NISHIZAKI Y, OHGA T, OHASHI Y, SATO T: Serum metabolomic profile and potential biomarkers for severity of fibrosis in nonalcoholic fatty liver disease. *J Gastroenterol* **48**: 1392-1400, 2013.

TRINH T, HARDEN NB, COLEMAN HM, KHAN SJ: Simultaneous determination of estrogenic and androgenic hormones in water by isotope dilution gas chromatography-tandem mass spectrometry. *J Chromatogr A* **1218**: 1668-1676, 2011.

VAN VYNCHT G, GASPAR P, DEPAUW E, MAGHUIN-ROGISTER G: Multi-residue screening and confirmatory analysis of anabolic steroids in urine by gas chromatography coupled with tandem mass spectrometry. *J Chromatogr A* **683**: 67-74, 1994.

VANKOVA M, HILL M, VELIKOVA M, VCELAK J, VACINOVA G, DVORAKOVA K, LUKASOVA P, VEJRAZKOVA D, RUSINA R, HOLMEROVA I, JAROLIMOVA E, VANKOVA H, KANCHEVA R, BENDLOVA B, STARKA L: Preliminary evidence of altered steroidogenesis in women with Alzheimer's disease: Have the patients "OLDER" adrenal zona reticularis? *J Steroid Biochem Mol Biol* **158**: 157-177, 2016.

VILJANTO M, PITA CH, SCARTH J, WALKER CJ, KICMAN AT, PARKIN MC: Important considerations for the utilisation of methanolysis in steroid analysis. *Drug Test Anal* **10**: 1469-1473, 2018.

VITKU J, HERACEK J, SOSVOROVA L, HAMPL R, CHLUPACOVA T, HILL M, SOBOTKA V, BICIKOVA M, STARKA L: Associations of bisphenol A and polychlorinated biphenyls with spermatogenesis and steroidogenesis in two biological fluids from men attending an infertility clinic. *Environ Int* **89-90**: 166-173, 2016.

WONG AS, LEUNG GN, LEUNG DK, WAN TS: Doping control analysis of anabolic steroids in equine urine by gas chromatography-tandem mass spectrometry. *Drug Test Anal* **9**: 1320-1327, 2017.

YAMADA M, ARAMAKI S, KUROSAWA M, KIJIMA-SUDA I, SAITO K, NAKAZAWA H: Simultaneous doping analysis of main urinary metabolites of anabolic steroids in horse by ion-trap gas chromatography-tandem mass spectrometry. *Anal Sci* **24**: 1199-1204, 2008.

ZUEHLKE S, DUENNBIER U, HEBERER T: Determination of estrogenic steroids in surface water and wastewater by liquid chromatography-electrospray tandem mass spectrometry. *J Sep Sci* **28**: 52-58, 2005.