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Review

Preclinical challenges in steroid analysis of human samples☆

Uta Ceglarek[a], Maria Werner[a], Linda Kortz[a], Antje Körner[b], Wieland Kiess[b], Joachim Thiery[a], Juergen Kratzsch[a,*]

[a] Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, Paul List-Str.13-15, Germany
[b] Hospital for Children and Adolescents, Liebigstrasse 20a, University Hospital, University of Leipzig, 04103 Leipzig, Germany

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ABSTRACT

Preclinical challenges in the analysis of steroid hormones are primarily determined by biological factors involved in the physiology and pathophysiology of hormone secretion. Major biologically influencing factors like age, sex, pubertal stage, pregnancy, phase of the menstruation, and diurnal rhythm have to be considered in the definition of reference ranges for steroids and their clinical interpretation. Hitherto, in clinical routine laboratories steroids were mainly determined by direct immunoassays applied on automated platforms, which are simple, rapid and cheap if a high number of samples are measured. However, technical factors like cross-reactivity of related steroid metabolites or limited analytical ranges have to be taken in account and may impair accuracy and precision of these direct methods. The actual development of mass spectrometry based analytical platforms for the determination of single steroid or steroid patterns seems to be an alternative analytical approach combining multi-parametric analysis, high sensitivity and specificity as well simple sample pre-treatment, robustness and low running costs for steroid analysis.

This short review will give an overview about biological influencing factors and technical disturbing factors of routinely used immunoassay for the analysis of steroids. The application of LC–MS/MS as an alternative routine high-throughput platform for steroid analysis and its perspective role in the standardization and harmonisation of steroid measurements in clinical routine application will be discussed.

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* Corresponding author at: Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital, Paul List-Str. 13-15, 04103 Leipzig, Germany. Tel.: +49 341 9722241; fax: +49 341 9722249.
E-mail addresses: kraj@medizin.uni-leipzig.de, juergen.kratzsch@medizin.uni-leipzig.de (J. Kratzsch).

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1. Introduction

Steroid hormones, synthesized in the adrenal cortex, gonads, placenta or other glands and tissues are of clinical importance for the diagnosis and monitoring of endocrine diseases and essential for infertility workup. A high number of biological and technical factors have to be considered in the analysis of steroids as may become evident as preclinical errors [1]. These preclinical errors are determined by the physiology of the steroid secretion dependent on chronobiological rhythms, menstrual cycle, age, in vitro pre-analytical factors like specimen collection or storage condition, and by the analytical method itself (e.g. specificity of the immunoassay).

A plethora of papers have been published about general requirements for steroid analysis using methods as competitive binding assay (CBA), indirect and direct immunoassay (IA), gas chromatography and tandem mass spectrometry (LC–MS/MS) [2]. This methodological development started between the sixties and eighties with the development of CBA and IA which were often coupled with preceding organic solvent extraction and chromatographic steps to enhance sensitivity and specificity of the analysis. At the same time gas/liquid chromatography methods have been established [3]. However, these techniques are rather time consuming and laborious. Faster direct immunoassays replaced the assays with pre-treatment in clinical routine analysis within the last 25 years. Although these direct immunoassays are simple, rapid and cheap, they may lack in reliability, sensitivity and specificity especially in low concentration ranges [4–6]. At present, the use of LC–MS/MS becomes increasingly important in clinical laboratory diagnostics. In the last decade an exponential growth in clinical laboratory, pharmacology, and toxicology applications of LC–MS/MS has been observed. The spectrum of MS applications ranges from the measurement of single analytes to qualitative and quantitative multi-parametric analyses [7]. The main advantages of mass spectrometry analysis over assays based on ligand binding are higher accuracy, specificity, shorter turnaround time and the capacity to perform quantitative multi-parametric analyses as demonstrated for steroid hormones and metabolites [8]. A sensitive multi-parametric approach was primarily described by Guo et al. for the analysis of 12 steroids in 11 min which required 200 μL serum [9,10]. Recently, we developed an on-line solid phase extraction (SPE)-liquid chromatography-triple quadrupole linear ion trap (LC-QTrap) method utilizing atmospheric pressure chemical ionization, whereby we were able to quantify simultaneously 9 steroids after protein precipitation of only 100 μL serum in 3 min. This new method was recently adapted for the use in our clinical routine lab [11]. In the near future it has to become evident if the analytical advantages of LC–MS/MS will lead to relevant new insights into to the physiology or pathophysiology of diseases that are associated with disturbances in steroid metabolism. Additionally, a substantial contribution of the LC–MS/MS methodology to the standardization and harmonisation of steroid measurements in clinical routine applications will be expected.

This short review will give an overview about biological influencing factors, technical disturbing factors and analytical challenges of routinely used immunoassays compared to LC–MS/MS for the analysis of estradiol, progesterone, testosterone, dehydroepiandrosterone-sulfate (DHEA-S), 17-hydroxyprogesterone (17-OHP), aldosterone and 25-hydroxy vitamin D (25(OH)D). These statements base partially on our first experience in the implementation of our alternative routine high-throughput platform for steroid analysis into the routine lab [11].

2. Biological influencing factors

Major biologically relevant influencing factors like age, sex, pubertal stage, pregnancy and phase of the menstruation are the basis for the establishment of reference ranges in laboratory diagnostics and therefore for the interpretation of clinical samples. In the following the potential effect of these influencing factors on steroid hormone concentration will be discussed.

2.1. Pubertal stage

For practical and physiological reasons gender-dependent puberty stages according to Tanner [12] or puberty-dependent age groups are the basis of reference ranges for fertility steroids of children and adolescents. However, glucocorticoids as cortisol and 11-deoxycortisol do not show a strong pubertal or age-dependence and therefore, reference ranges could be given for the whole group of children independently on the variety of age. Our results for the establishment of reference ranges for serum cortisol, 17-OHP, progesterone, 11-deoxycortisol, testosterone, DHEA-S and androstendione support this suggestion. We measured the steroids as an LC–MS/MS profile [11] from 200 healthy children with a defined Tanner stage estimated by experienced pediatric endocrinologists. The findings presented in Tables 1a and 1b confirm the pronounced pubertal increase in steroids in dependence on gender with exclusion of 11-deoxycortisol. Differences in the strength of the associations between hormone levels and influencing factors are summarized in Table 2. Testosterone and androstendione were highly significant correlated to puberty or age. In contrast to the data of these fertility steroids, we found only a minor puberty-dependent association with progesterone and cortisol as well as no significant data for the 11-deoxycortisol levels. Additionally, we could not reveal any sex specific differences for both hormones in the individual Tanner stages. Changes of estradiol during puberty were found to be similar in girls and boys [13]. Taken together, our results point out distinct differences in the association between individual steroid parameters and pubertal stage in children and adolescents.

2.2. Menstruation cycle and pregnancy

In adults female levels of estradiol, progesterone and testosterone vary across the menstruation cycle [14]. Therefore, knowledge about details in this physiological rhythm is important for establishing reference ranges and for interpretation of clinical data. The pituitary gonadotropic hormones FSH (follicle-stimulating hormone) and LH (luteinizing hormone) are involved in the regulation of ovarian steroid synthesis. In the follicular phase plasma estradiol and progesterone concentration rises. After the midcycle both of them show a second peak in their plasma concentration in the luteal phase [15]. Ovarian androgens levels rise

Table 1a
Reference intervals (5–95 percentile) for steroid measurements by LC–MS/MS (Ceglarek et al. [11]) in serum of boys in dependence on Tanner stage or age.

	N	TESTO (nmol/l)	ANDRO (nmol/l)	PROG (nmol/l)	17-OHP (nmol/l)	11-DC (nmol/l)	CORT (nmol/l)	DHEA-S (μmol/l)
P								
1	26	0.07–0.29	0.14–0.63		0.26–1.89		89–345	0.21–1.88
2	18	0.29–13.0	0.46–1.48		0.27–3.03		68–298	0.82–4.15
3	15	2.81–23.9	0.72–2.20		0.54–2.22		88–293	1.90–5.21
4	16	5.90–23.3	0.83–3.36		1.02–2.53		79–351	1.66–7.65
5/6	24	7.50–20.0	0.84–2.47		1.36–4.03		120–392	1.23–7.08
1–6	99			0.10–0.41		0.54–4.80		
AG								
7–9	19	0.07–0.21	0.14–0.52	0.10–0.21	0.25–1.48		62–248	0.17–1.88
10–12	22	0.16–7.74	0.54–1.16	0.10–0.52	0.58–2.32		101–345	0.73–4.15
13–14	31	2.72–20.7	0.62–2.19	0.10–0.53	0.31–2.98		79–345	1.43–4.34
15–17	27	4.79–23.3	0.99–3.07	0.10–0.36	1.14–3.18		145–351	1.57–7.54
7–17	99					0.54–4.80		

Abbreviations: TESTO for testosterone; ANDRO for androstendione; PROG for progesterone; 17-OHP for 17-hydroxyprogesterone; 11-DC for 11-deoxycortisol; CORT for cortisol; DHEA-S for dehydroepiandrosterone-sulfate; P, Tanner stage; AG, age group (years).

during the follicular phase, too. After a peak in the midcycle they decreased during the luteal phase till the next follicular phase. For free and total testosterone similar results were observed [14,16]. A preovulatory increase of testosterone 3 days before the LH peak was observed [17]. Respective changes of fertility hormones were also demonstrated in pregnancy or with the beginning of the menopause. Menopausal women had lower estradiol, progesterone [18] and testosterone [19] levels than normal cycling women. In contrast, no significant difference has been shown for 25(OH)D if serum levels of postmenopausal and premenopausal women were compared [20].

2.3. Age and gender

Adult males and females demonstrate a clear age-dependent decrease in testosterone, DHEA-S and androsterone [21–23] but also in progesterone [24] and aldosterone [25,26]. In particular, female serum androgen levels decline steeply in the early reproductive years and do not vary because a consequence of natural menopause as the postmenopausal ovary appears to be an ongoing site of production for testosterone and its metabolites. These significant variations in androgens related to age must be taken into account when reference ranges are reported [27]. Beyond that, the strong gender-dependence in the levels of reference ranges for testosterone, estradiol, and progesterone has to be considered [18]. Also 25OHD levels have been reported to decline with age and be lower in women than men [28]. Unfortunately, manufacturers of immunoassay kits do mostly not provide age-dependent hormone levels for their established reference ranges. In contrast to fertility steroids and androgens, recent literature demonstrated no clear age- and sex-dependence for cortisol or 11-deoxycortisol in adults [29].

2.4. Diurnal rhythm

In males, the diurnal rhythm of adrenocorticotropic hormone (peak before awaking, decline during day progress) is a reflection of neural control and provokes concordant diurnal secretion of cortisol and other minor ACTH-dependent adrenal steroids [30]. Within the reproductive axis hormone secretion is determined by the episodic nature of gonadotropins and of gonadal steroid hormone response to these pulses. This regulation mechanism overshadows a potential diurnal variation except in adolescents. Moreover, sexual hormone-binding globulin (SHBG) may affect steroid hormone levels in blood. Variations in SHBG binding capacity appeared to be associated with the changes of total testosterone, free testosterone and testosterone/SHBG index, which showed also the highest concentrations in the morning and the lowest levels in the evening during the 24 h test period. However, the percentage free testosterone remained unchanged [31]. Other reproductive steroids as progesterone, pregnenolone and 17-OHP exhibited a time course of plasma concentrations assuming a regulation predominantly dictated by the testicular secretory activity as well [30]. Much lower levels of diurnal variation compared to the variation of glucocorticoids and androgens were found for estradiol [32].

Females demonstrated during the normal menstruation an overlap between diurnal and episodic secretion in estrogens and progestens whereas in androgens and glucocorticoids only minimal fluctuations were seen [33]. Thereby, no significant diurnal changes of SHBG binding capacity, total estradiol, free estradiol,

Table 1b
Reference intervals (5–95 percentile) for steroid measurements by LC–MS/MS (Ceglarek et al. [11]) in serum of girls in dependence on Tanner stage or age.

	N	TESTO (nmol/l)	ANDRO (nmol/l)	PROG (nmol/l)	17-OHP (nmol/l)	11-DC (nmol/l)	CORT (nmol/l)	DHEA-S (μmol/l)
P								
1	37	0.07–0.33	0.21–1.01	0.10–0.82	0.26–3.03		61–328	0.27–3.04
2	12	0.17–0.58	0.61–1.89	0.10–0.24	0.33–1.22		91–218	0.44–2.85
3	12	0.15–1.18	0.97–4.64	0.10–0.20	0.52–1.98		90–428	0.61–3.12
4	12	0.24–1.01	0.96–2.85	0.10–24.0	0.41–3.51		51–665	0.56–5.32
5	27	0.37–1.48	1.20–3.98	0.10–19.0	0.28–2.59		109–420	1.17–7.06
1–5	100					0.49–5.10		
AG								
7–9	31	0.07–0.32	0.21–0.91	0.10–0.82	0.29–3.03		92–328	0.27–3.04
10–12	30	0.14–0.70	0.57–2.00	0.10–0.20	0.32–1.51		51–293	0.44–2.85
13–14	17	0.36–1.50	1.05–5.55	0.10–24.0	0.57–3.51		109–665	1.02–3.31
15–17	22	0.36–1.01	1.11–3.02	0.10–19.0	0.28–2.57		115–420	1.05–7.06
7–17	100					0.49–5.10		

Abbreviations: TESTO for testosterone; ANDRO for androstendione; PROG for progesterone; 17-OHP for 17-hydroxyprogesterone; 11-DC for 11-deoxycortisol; CORT for cortisol; DHEA-S-for dehydroepiandrosterone-sulfate; P, Tanner stage; AG, age group (years).

Table 2
Coefficients of correlation according to Spearman for the associations between serum steroid levels of healthy children (boys $n = 99$; girls $n = 100$) measured by LC–MS/MS profiles and tanner stage, age and gender.

Analyt	Independ. variable	Pearson correlation coefficient *R*		
		All subjects	Boys	Girls
TESTO	Tanner stage	0.745***	0.881***	0.809***
	Age group	0.765***	0.801***	0.799***
	Sex	0.447***		
ANDRO	Tanner stage	0.812***	0.807***	0.826***
	Age group	0.776***	0.815***	0.800***
	Sex	−0.090		
PROG	Tanner stage	0.275***	0.190	0.326***
	Age group	0.337***	0.263**	0.353***
	Sex	0.078		
17-OHP	Tanner stage	0.480***	0.669***	0.298**
	Age group	0.472***	0.635***	0.244*
	Sex	0.290***		
11-DC	Tanner stage	−0.033	−0.031	−0.039
	Age group	0.033	0.079	−0.038
	Sex	0.064		
CORT	Tanner stage	0.266***	0.251*	0.260**
	Age group	0.310***	0.316**	0.248*
	Sex	0.116		
DHEA-S	Tanner stage	0.620***	0.597***	0.618***
	Age group	0.682***	0.679***	0.612***
	Sex	0.197**		

Abbreviations: TESTO for testosterone; ANDRO for androstendione; PROG for progesterone; 17-OHP for 17-hydroxyprogesterone; 11-DC for 11-deoxycortisol; CORT for cortisol; DHEA-S for dehydroepiandrosterone-sulfate.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

percentage free estradiol and percentage free testosterone were found in the mid-luteal phase, although significant fluctuations of total testosterone, free testosterone and testosterone/SHBG index were observed throughout the day [31].

During puberty, girls revealed a marked diurnal rhythm of estradiol and testosterone with high levels in the morning, for boys a similar pattern was found in late puberty [34–36]. If measured by a bioassay estradiol levels appeared to have a through occurring 8.00–20.00 h in girls and 12.00–20.00 h in boys [37] suggesting a potential method dependency of the analytical results. Increased morning adrenal steroids like cortisol, aldosterone, DHEA-S, and androstendione demonstrated even a more pronounced diurnal rhythm in children and adult age [38]. Generally, time of blood withdrawal is important for the establishment of reference ranges for the majority of steroids and for the interpretation of their clinical data.

2.5. Seasonal variation

Seasonal variation significantly affects the diagnosis of vitamin D sufficiency, which requires seasonally adjusted thresholds individualized for different locations. Clinicians should consider the month of sampling and the amount of body fat when interpreting 25(OH)D measurements [39].

2.6. Physical or mental stress

A large variety of stressors can rapidly affect the adrenal cortex via the adrenocorticotropic axis. Thereby, pituitary derived ACTH triggers the secretion of glucocorticoids, mainly cortisol from the adrenal cortex. Thus, the presence of stressors should be avoided during blood withdrawal in general. Alternatively, saliva represents a biological fluid that allows a stress-free sampling of free steroids and reflects the physiological and pathophysiological status of the adrenocorticotropic axis [40,41].

Characteristic features of physical or mental stress are increased levels of corticoids [42–44] and decreased levels of testosterone [42,45,46]. Even an interview derived decreased self-rated health score below the level of good was associated with significantly decreased serum testosterone [47]. Negative associations between the psychosocial working environment and testosterone were described [48].

During critical illness, glucocorticoid secretion markedly increases. However, this increase is not discernible when only the serum total cortisol concentration is measured [49]. In this study, subnormal serum total cortisol concentrations were found in nearly 40% of critically ill patients with hypoproteinemia, even though their adrenal function was normal. Measuring serum free cortisol concentrations may help to gain physiologically relevant data from these patients. Hypotestosteronaemia was found in the majority of men and was significantly associated with severity of illness [50]. In contrast, estrogen levels rose due to increased peripheral aromatization [51].

2.7. Fasting

The issue whether or not fasting is recommended before blood withdrawal in the measurement of steroids is not fully validated for each parameter so far. Fasting appeared to have no acute effect on the fertility steroid estradiol [52], but progesterone was observed to be somewhat lowered after a balanced liquid meal in women [53]. Adrenal steroids were rather be influenced by the absence or presence of food. Cortisol [54] and DHEA-S [55] levels were increased by longer fasting and additionally, cortisol levels were elevated by an ingested meal [53]. In contrast, cortisol was found to be reduced after carbohydrate intake [56] leading to the suggestion that the type of nutrition may also affect hormone levels. Also testosterone was determined to be significantly lower over 48 h fasting [57]. To guarantee the reproducibility in the measurement and to exclude any effect of nutrition intake, withdrawal of blood in fasting state would be desirable in steroid analytics.

2.8. Body mass

Body mass is a further potential influencing factor on steroid levels in healthy subjects. Thus, in the post- but not in premenopausal women, estrogens, testosterone and androstendione increased with growing BMI [58]. In males, BMI and testosterone levels [59] were shown to be inversely correlated. Another study revealed that DHEA-S levels were lower in an obese group than in lean premenopausal women. For all these subjects, DHEA-S levels were negatively related with BMI and hip circumference [60]. A comparable negative correlation was also found in a whole-age study investigating vitamin D in females [61]. All these observations offered evidence that obesity may influence the levels of endogenous steroids, especially after menopause. This suggestion has to be considered in the interpretation of patient findings but the strength of these relationships was limited and did not justify the establishment of BMI-dependent reference ranges.

3. Technical factors

If the literature about technical factors that potentially influence analytical results is reviewed it becomes clear that most of the previous findings were revealed by immunoassay. As respective LC–MS/MS data are still scarcely available, the generalization of the knowledge deduced from immunoassay should be done care-

fully, as the effect of technical factors may depend on the analytical principles of the measuring method.

3.1. Use of different blood matrices

Steroid levels determined from some types of plasma may be found in marginally different levels compared to serum [62–66]. Other data from the literature documented no significant difference between serum and plasma levels of estradiol, estrone, progesterone, testosterone and cortisol [66]. No significant differences in analyte recovery for serum, EDTA, or heparin plasma were found for 11-deoxycortisol, 17-OHP, and 17-OH pregnenolone by LC–MS/MS [67]. However, the same group detected a 280% higher level of pregnenolone if sodium EDTA was used instead of serum or heparin plasma by the same method.

3.2. Type of tube for blood withdrawal

For estradiol [68], cortisol [69], progesterone [69], DHEA-S [68] values of specimens collected in solid sealing technology (SST) glass tubes were found significantly different or scattered compared to specimens collected in plain glass tubes. In another study, no differences of cortisol were measured in 4 different vacutainers types [70]. Accordingly, the selection of a special type of tube appears to have minor relevance for steroid measurements,

3.3. Storage stability of samples

Steroids were found to be relatively stabile in plasma and serum [71–74], but also in whole blood [71]. Moreover, serum testosterone and cortisol levels appeared even to be unchanged over a time period of 40 years at −25 °C [75]. DHEA-S demonstrated a decrease in concentration after 2 days at room temperature [65]. Interestingly, Kushnir et al. [67] observed that serum levels of 11-deoxycortisol and 17-OHP were stable in the LC–MS/MS determination for at least 10 days storage at room temperature whereas pregnenolone or 17-OH pregnenolone levels decreased even after a couple of days. However, all four steroids remained stabile during 3 months of storage at −20 °C. Stability data of estradiol and estrone from LC–MS/MS were comparably documented by unchanged measuring values over at least 2 months at −70 °C in the freezer or at refrigerator storage at 4 °C [13].

3.4. Freeze–thaw stability of samples

Although manufacturer of the steroid assay alert for multiple freeze/thaw cycles to avoid the deterioration of the analyte many papers describe stabile steroid levels after a definite number of cycles: 10 freeze and thaw cycles lead to no relevant changes in levels of DHEA-S, cortisol, 17-OHP and aldosterone [71]. This finding was confirmed for 12 freeze and thaw cycles of dihydrotestosterone, testosterone, estradiol, and progesterone [76]. Serum 25(OH)D demonstrated no relevant change in its level after 4 freeze and thaw cycles [77], but lithium heparin plasma was only stabile without freezing [78]. First LC–MS/MS results proved that estradiol and estrone may be undergone 3 freeze and thaw cycles without losing analytic signal in their measurement [13]. However, each method and sample material has to be validated for the use of samples with a disrupted cold chain.

3.5. Type of sample matrices

The determination of steroids has been usually performed in the centrifuged serum or plasma supernatant of blood samples. As steroids are relatively stable against changes in pH or molarity of their biological fluid, urine is a further relevant sample matrix that enables to determine time-independent levels of steroids by urine collection over the whole day. In the clinical routine analysis urinary cortisol and aldosterone measurements play the most important role [79,80]. Before urine samples are measured by immunoassay they have to be extracted for the separation of unwanted interfering substances and to concentrate for fitting the lower urine levels compared to serum to the optimal measuring range of the assay. Urine steroids were also relatively stabile in their storage, for LC–MS/MS the stability of testosterone and epitestosterone was shown for at least 22 months [81].

The small size of steroid molecules enables their entering into the saliva compartment from peripheral blood by passive diffusion. These salivary levels reflect unbound steroids in the range of approximately 1% of the concentration in serum or plasma. Especially measurement of salivary cortisol in the diagnostic of Cushing's disease, and the determination of 17-OHP in monitoring the compliance in the congenital adrenal hyperplasia are well established in routine analysis. A circumstantial overview about steroid analysis in saliva was published by Gröschl in 2008 [82].

Moreover, it should be kept in mind that the level and the characteristics of cross-reacting substances is completely different in urine or saliva compared to serum or plasma. Consequently, specific assay validation for the use of such sample matrices is mandatory, independent on the type of the analytical method that had been applied.

4. Analytical factors

4.1. Interference by cross-reactive substances

Potential interference of non-target but structure-related endogenous secreted or exogen-added steroids or steroidal drugs may play an important role for the specificity of analytic measurement. In the establishment of new LC–MS/MS procedures there is a chance to simply validate potential interference on the particular method. Thus, Kushnir et al. [13] investigated the interference effects, by adding 100 μg/L of 50 structure-related steroids to the estrogens estrone and 17β-estradiol. Only one of these substances, the isomere 17α-estradiol, interfered with the target steroid due to co-elution from the HPLC-column. However, independently on the issue whether or not the interference of 17α-estradiol isoform could be clinically relevant, it would be possible to completely separate the 17α isoform from the target by modifying the chromatographic conditions. In contrast, in a well working immunoassay for 17β-estradiol, 34 structure-related substances were extensively characterized for their interference with the analyte [83] but a potential interference of 17α-estradiol was not tested. However, it can be assumed that the structural similarity of 17α-estradiol with 17β-estradiol results in high cross-reactivity of this molecular form. However, the methodology of immunoassays allows only the separation of molecular isoforms of the target antigen, not recognized by differences in the antigen–antibody interaction, chromatographic or solvent extraction methods were applied before the analysis. Moreover, the presence of one hydroxyl- or keto-group more or less in a structure-analogue molecule can but must not necessarily lead to considerable analytical interference that consequently results in falsely increased levels of the target hormone by immunological methods. A relevant example for this issue is also the measurement of cortisol by direct immunoassay. Whereas a LC–MS/MS method completely enabled the separation of the structural related parameters cortisol, cortisone, prednisolone and prednisone by the help of specific LC column [84], antibodies of recent direct immunoassays recognized at least relevant levels of prednisolone as cross-reactive substance [85,86]. To overcome this non-specificity again a pre-

posed LC step for the isolation of cortisol from the interfering substances before the immunoassay or an improved epitope specificity of the antibody would be helpful. However, both additional analytical steps would be extremely cumbersome and expensive or could reduce analytical quality and efficacy of the test as in particular by the pre-posed LC step. This point leads to the conclusion that potential interference must be extensively tested for each analytical method and clinical application, but the use of LC–MS/MS methods may improve a lack in specificity of immunoassays by involving additional analytical tools as especially different pre-columns.

4.2. Interference by hemolysis and lipemia

Hemolysis or lipemia can be easily optically recognized and may have impact on steroid levels by suppressing the analytical signal. Accordingly, severe hemolysis or lipemia should be generally avoided. Thus, 500 mg/dL or 1000 mg/dL haemoglobin leads to approximately 13% [87] or <10% [83] lowered estradiol concentrations in dependence on the used immunoassay method. Lipids at levels of less than 1000 mg/dL caused only a minor reduction of 10% in estradiol levels [83].

Comparable decreases were observed for testosterone by a concentration of 500 mg/dL haemoglobin [88]. In contrast, respective level of haemoglobin or lipids had a higher ($\gg$10%) effect on testosterone measured by another immunoassay method [89]. Accordingly, a high degree of hemolysis or lipemia in blood should be avoided, and if present the detailed effect has to be checked for the individual method. This recommendation is also appropriate for the issue whether or not hemolysis or lipemia may have effects on signals of LC–MS/MS measurement.

4.3. Measurement of free hormone levels in blood

The majority of steroid molecules are bound to high affinity binding proteins as sexual hormone-, corticosteroid- or vitamin D-binding protein but also to low-affinity proteins like albumin and pre-albumin. As free hormones circulate in blood only in the range of 1% compared to the levels of the total analyte a more sensitive and specific detection method is necessary. At least the measurement of free testosterone appears to better reflect the biological activity and is therefore available in clinical laboratories as immunoassay method. However, the separation of the free from the total amount of this steroid is a very complex and tricky procedure in routine analysis and lead often to an over- or underestimating of the expected result in immunoassay [90]. The use of LC–MS/MS methods for the determination of free steroids would require a complete separation of this sample component in a pre-treatment step and its subsequent measurement. It remains unclear if sensitivity and precision of LC–MS/MS will be high enough and the pre-treatment procedure will be practicable enough to measure free steroids also by this method. Alternatively, the mathematical calculation of free hormone levels after determination of binding protein and total hormone levels and involving their association constants has been shown to be a valuable alternative for their direct determination [91].

5. Summary

A number of biological and technical influencing factors have to be considered as preclinical challenges before establishing methods or interpret findings from steroid levels in biological fluids.

- Gender-dependent puberty stages or puberty-dependent age groups are the basis of reference ranges or fertility steroids of children and adolescents.
- In adults females fertility steroid concentrations vary across the menstruation cycle, pregnancy or with the beginning of the menopause. Adult males demonstrate an age-dependent decrease in the majority of steroids and a reference range distinctly different from female levels.
- Time of blood withdrawal is generally important for the establishment of reference ranges in the majority of steroids and for the interpretation of their clinical data.
- To guarantee the reproducibility of the measurement and to exclude any effect of nutrition intake withdrawal of blood in fasting state would be desirable.
- Potential interference must be extensively tested for each analytical method and clinical application, especially if new procedures as tandem-MS/MS are used.
- Blood samples for steroid analytics should be stored as serum in the frozen state.
- Specific assay validation for the use of sample matrices different from serum is mandatory.
- For sample retrieval severe hemolysis or lipemia should be generally avoided.
- Highly sensitive immunoassays are widely used in clinical laboratories for the measurement of very low concentrated free steroids in serum. The application of LC–MS/MS would require a previous separation and pre-concentration step in this case. Combining immunochemical separation with mass-spectrometric detection may help to overcome the current analytical limitations in the future.

Additionally, a number of analytical and clinical studies have to be done yet to confirm or newly evaluate the whole body of preclinical challenges for the use of LC–MS/MS in steroid analytics for the routine diagnostic.

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