Chapter I

General introduction
The measurement of hormone concentrations is vital for clinical endocrinology as well as endocrine research. Since the beginning of the 21st century increased attention has been paid to the quality of hormone measurements, especially in steroid hormone analysis. The origin of the present thesis is by the publication of Taieb et al. in 2003, in which it was shown that commonly used immunoassays for testosterone were not suitable to measure the low testosterone concentrations in females and children [1]. The immunoassays investigated in this study were found to be comparable or even worse than a random number generator in estimating serum testosterone levels in females [2]. This publication was followed by an extensive debate on the quality of testosterone assays, which finally resulted in Endocrine Society's Position Statement and the subsequent Consensus statement on testosterone assays. These statements underlined the urgent need to improve the accuracy of testosterone measurements [3,4]. Ever since much effort has been put in the development and validation of accurate testosterone assays.

Testosterone is one of the many steroid hormones. Steroid hormones form a group of hormones, mainly produced in the adrenal and gonadal glands. Steroids are produced from cholesterol and undergo many enzymatic conversions before active hormones are formed. There are four groups of steroid hormones: glucocorticosteroids, mineralocorticosteroids, neurosteroids and sex steroids. Steroids may belong to more than one group, as exemplified by progesterone for instance. Glucocorticosteroids (e.g. cortisol) and mineralocorticosteroids (e.g. aldosterone) are produced in the adrenal glands and have their function in metabolism, inflammation and stress adaptation; and salt and water homeostasis, respectively. Neurosteroids are produced in the central nervous system. The group of sex steroids consists of a group of precursor hormones such as dehydroepiandrosterone (DHEA) and its sulphate (DHEAS) as well as androstenedione, and biologically active hormones such as testosterone, dihydrotestosterone and estradiol. Sex steroids are important for primary and secondary sexual development, sexuality, fertility and bone and muscle metabolism. Sex steroids are synthesized in the adrenal as well as the gonadal glands and released to the circulation either as precursor or as active hormone. The precursor sex steroids are able to undergo enzymatic conversion in their target tissues, such as skin, central nervous system, hair follicles, bone and muscles [5]. This thesis focuses on the male sex steroids: androgens and their precursors (testosterone, DHEA, DHEAS, and androstenedione). DHEA and DHEAS as well as androstenedione are androgenic precursors of the biologically active male and female sex steroids testosterone, dihydrotestosterone and estradiol. DHEA is mainly produced in the adrenal glands, whereas androstenedione is produced by the adrenals as well as the gonads [5,6]. Testosterone is produced in the gonadal and the adrenal glands as well as converted from androstenedione in peripheral tissues. For diagnostic purposes androgen precursors, such as DHEA, DHEAS and androstenedione are mainly measured in case of suspicion of adrenal tumours, adrenal insufficiency, congenital adrenal hyperplasia, or other adrenal diseases [6,7]. Androstenedione as well as testosterone are often determined in male and female subfertility, polycystic ovary syndrome and other disorders of the menstrual cycle, or in men in case of suspicion of hypogonadism as well as during the follow up in prostate cancer [6]. The simultaneous determination of multiple androgens
can be helpful in the clinical workup of androgen excess in children and females as well as in other androgen related diseases and in several research settings.

Quantitative measurement of steroid hormones is complex. This is mainly due to their structural similarity, which can cause cross reaction in assays [8;9], as well as the very low concentrations in which some steroids, such as testosterone in children and females, are present in humans. In general, the techniques available for steroid analysis can be divided into two groups: competitive (radio-)immunoassays ((R)IA) and mass spectrometry based methods (gas-chromatography-mass spectrometry (GC-MS) and Liquid-Chromatography Tandem Mass Spectrometry (LC-MS/MS)). GC-MS is considered the gold standard for quantitative analysis of steroids. Gas chromatographic separation of steroids is very reproducible and accurate [10] and leads to the possibility to measure several steroid hormones in one assay. GC-MS methods usually require large sample volumes, an extensive sample preparation including addition of internal standards, extraction and derivatization and have limited throughput due to long analyses. Moreover, handling of a GC-MS requires highly trained technicians. For these reasons, GC-MS is usually not used for routine diagnostic or research purposes. For long times, radioimmunoassays in combination with liquid-liquid extraction and chromatographic purification were used for steroid hormone analysis. The accuracy of these assays, if properly validated, was comparable to GC-MS. However, when more specific antibodies became available and quick throughput became important, the extensive sample preparation using extraction and chromatography was step by step abandoned. Currently, many laboratories use (automated) direct immunoassays for steroid analysis. Leaving out purification steps has led to problems such as loss of specificity and precision. Taieb et al. showed that the immunoassays used in the early 2000s were not suitable for testosterone measurement in females and children [1]. Although the second generation immunoassays for testosterone are better, this is still a problem [11]. LC-MS/MS has become available in clinical laboratories in recent years. If developed and validated well, an LC-MS/MS method has comparable accuracy, precision and sensitivity as GC-MS [12;13]. Furthermore, also LC-MS/MS offers the possibility of simultaneous measurement of multiple steroids. Usually, sample preparation in LC-MS/MS analysis includes internal standard addition and some kind of sample purification (e.g. protein precipitation, liquid-liquid extraction, solid-phase extraction) and sometimes derivatization to increase sensitivity. The chromatographic separation is in LC usually shorter when compared to GC. Tandem mass spectrometry (MS/MS) has the advantage of two selection criteria (parent and daughter ion), which increases specificity. Despite these advantages, LC-MS/MS, like GC-MS, is a complex technique and requires highly trained technicians. Motivated by the superiority in specificity over immunoassays, several laboratories have now adopted LC-MS/MS for steroid hormone analysis.

During the last years many papers on newly developed LC-MS/MS methods for steroid hormone measurement have been published [14]. Chapter II of this thesis describes the characteristics and performance of an LC-MS/MS method for the quantitative determination of DHEAS in serum and cerebrospinal fluid. Chapter IV describes an LC-MS/MS method for the simultaneous quantitative measurement of testosterone, androstenedione and DHEA in serum and
plasma. The development and availability of LC-MS/MS assays for the analysis of steroid hormones, together with its promise of more accurate steroid hormone measurement has prompted the Editorial Board of the Journal of Clinical Endocrinology and Metabolism (JCEM) to state that from 2015 on ‘manuscripts reporting sex steroid assays as important endpoints must use MS-based assays’ and ‘it is anticipated that [this requirement] will be extended to adrenal steroids and vitamin D in the near future’ [15]. Although the requirement for reliable steroid assays is urgent and the superiority of LC-MS/MS above immunoassays seems to be clear, the JCEM statement raises several questions. Firstly, it was not yet shown that immunoassays are unreliable for all steroid hormones. A comparison between the currently available immunoassays and an LC-MS/MS method for the determination of DHEAS serum concentrations has never been published. Chapter III of this thesis investigates the performance of seven currently used immunoassays for measurement of serum DHEAS concentrations in comparison to the LC-MS/MS method described in Chapter II.

Secondly, there is little information available on the mutual agreement of LC-MS/MS assays. For testosterone, it was shown that LC-MS/MS assays for testosterone may have a high precision and agree well with each other and a reference method [13]. However, other studies show a much higher variation and a less strong agreement between LC-MS/MS methods for testosterone [16;17]. Data on the agreement of LC-MS/MS assays for adrenal steroids are even scarcer. The studies described in Chapter V and Chapter VI of this thesis aim to provide these data. Chapter V describes a comparison between seven published LC-MS/MS methods for the simultaneous analysis of serum testosterone, androstenedione and DHEA concentrations and Chapter VI describes a comparison between eight routine unpublished LC-MS/MS methods for the simultaneous measurement of serum testosterone and androstenedione concentrations and the method described in Chapter IV.

Thirdly, since 2003, when Taieb et al. published their comparison study for testosterone immunoassays, a new generation of automated immunoassays for testosterone has become available. In general, the currently used 2nd and 3rd generation testosterone assays show a clear improvement in the lower concentration range when compared to the first generation assays investigated by Taieb et al. [1;11;18]. Therefore, the performance of the currently used immunoassays should be taken into account when investigating the potential superiority of MS-based testosterone assays. Data on the variation of currently used testosterone immunoassays in comparison with LC-MS/MS assays was lacking. In Chapter V and Chapter VI, the variation among the investigated LC-MS/MS testosterone assays was compared to the variation among the currently used immunoassays for measurement of testosterone.

The second half of this thesis focuses on the measurement of testosterone and androstenedione in saliva. Steroid hormone levels in saliva are much lower than in serum (19). Therefore, assays for salivary steroids need to be as highly specific and even more sensitive than assays used to determine serum steroid levels. Chapter VII describes three LC-MS/MS methods including a method comparison for the simultaneous measurement of testosterone and androstenedione concentrations in saliva.
The measurement of steroid hormones in saliva, is an attractive alternative to serum analysis mainly because of the simple and non-invasive sample collection. Subjects can easily collect their saliva samples themselves by drooling their saliva in a simple polypropylene tube. This needs no special training, is also suitable for older children as well as sampling at home or in field studies [20]. Moreover, it gives the ability of repeated sampling in cases where several venipunctures are impossible or undesirable. Salivary steroid hormone concentrations are thought to reflect serum free steroid concentrations [21;22]. Salivary steroid analysis is therefore used to diagnose and monitor patients with alterations in androgen levels as well as a measure to investigate the relationship between hormone biology and many aspects of human life, such as psychology [23]. Salivary testosterone is often requested in clinical diagnosis. In children and adolescents testosterone is mainly measured to investigate whether puberty has started or not as well as the follow up of testosterone therapy [24;25]. To be able to interpret salivary testosterone concentrations reliable and method dependent reference values have to be available. Chapter VIII describes reference values for salivary testosterone determined in adolescent boys and girls aged 8 to 26 years determined using an LC-MS/MS method.

In clinical and research daily practice several different ways of saliva sample collection are used. The most straightforward way is to collect unstimulated, total saliva by waiting until saliva is produced and drooling in a tube. However, some subjects have a very low passive saliva production. Therefore, often stimulated saliva is used for analysis. Saliva secretion can be stimulated in several ways, but is usually stimulated by masticatory action (e.g. chewing chewing gum or Salivette® swabs (Sarstedt, Nümburg, Germany)) or by gustatory stimuli (e.g. oral application of citric acid). However, the concentration of some steroid hormones might be influenced by the salivary flow rate, most probably depending on the way they are excreted. Only free lipid-soluble unconjugated steroids like testosterone and cortisol are able to pass into saliva by the diffusion route (intracellular route). It is hypothesized that they pass through the capillary wall, basement membrane, and acinar cells of the secretory end pieces of the salivary glands along a concentration gradient and enter the saliva. In contrast, the lipid-insoluble conjugated steroids like dehydroandroepiandrosterone-sulphate (DHEAS) are thought to enter saliva via the tight junctions between the acinar cells (the ultrafiltration route) [22]. Vining et al. found that cortisol concentration were equal in unstimulated and stimulated parotid saliva, suggesting that the intracellular excretion route of cortisol is not significantly influenced by the salivary flow rate. In contrast, in the same study DHEAS concentrations were found to be much lower in stimulated parotid saliva when compared to passive parotid saliva. This suggests that the excretion of DHEAS via the ultrafiltration process is highly dependent of the salivary flow rate [22]. As clinicians and researchers often do not distinguish between unstimulated and stimulated saliva, data to make sure whether or not both types of saliva can be used interchangeably for steroid analysis are needed. In addition under stimulated conditions, saliva is mainly produced by the parotid glands, whereas the other salivary glands are mainly responsible for the basal saliva production [26]. Little is known about a possible difference in steroid hormone excretion by the different salivary glands. Whether the unconjugated steroids testosterone, androstenedione and cortisone are also excreted independent of the salivary flow rate was described in Chapter IX. In the
same Chapter the influence of several methods to stimulate saliva production and collection from different glands on the concentration of salivary testosterone, androstenedione, cortisol an cortisone levels was described.

The importance of a reliable pre-analysis and analysis of steroid hormones in saliva was highlighted in Chapter X of this thesis.

Finally, in Chapter XI, the findings of the studies presented in this thesis are summarized and discussed in the perspective of the requirements for accurate measurement of adrenal steroid hormones in serum and saliva.
References


