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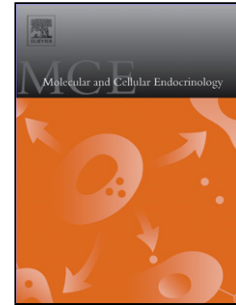
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## Steroid measurement with LC-MS/MS in pediatric endocrinology

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**Abstract:** The liquid chromatography tandem mass spectrometry (LC-MS/MS) is an increasingly common tool in the clinical laboratory. Established applications include routine assays for detecting inborn errors of metabolism and for monitoring therapeutic drugs and steroids. Steroid profiling is a very effective method for distinguishing almost all steroid related disorders. It allows accurate diagnosis and is very useful in many clinical situations. Most methods for the determination of steroid hormones are based on immunoassays, which are rapid and easy to perform. However, the reliability of steroid immunoassays has been shown to be doubtful because of the lack of specificity and of matrix effects. Immunological methods, especially direct assays, often overestimate true steroid values. This is of particular importance in the newborn period and in early infancy. Problems with steroid immunoassays have further been reported for female patients or when analysing different media, e.g. saliva. Patient follow-up over time or between laboratories, as well as longitudinal studies are extremely difficult. In contrast to immunoassays, which allow the measurement of only a single steroid at a time, LC-MS/MS has the advantage that a wide spectrum of steroid hormones can be measured simultaneously. The applicability for clinical samples and problems in pediatric endocrinology will be discussed.

## 1. Introduction:

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an increasingly important analytical technology in the clinical laboratory environment (Dooley, 2003; Vogeser and Seger, 2008). One of the first applications was multiple analyte screening for inborn errors of metabolism (Chace et al., 1993; Chace et al., 2003). This application embodied one of the seminal advantages of LC-MS/MS, that is, the ability to quantitate a large number of analytes in a single scalable measurement process. Another major stimulus for its increased use has been the appearance of new immunosuppressive drugs and the development of multiple-drug therapeutic regimens (Ceglarek et al., 2006; Koal et al., 2004; Streit et al., 2002; Taylor, 2004). We are now seeing a vast array of new applications being developed, such as peptides and proteins (Bondar et al., 2007; Rauh et al., 2007). Recently, a number of LC-MS/MS-based methods using different ion sources have been reported for the determination of steroids (Vogeser and Parhofer, 2007).

### 1.1 Steroid disorders in children

Serum steroid assays play an important role in the clinical evaluation of a number of common endocrine disorders (Holst et al., 2004). The correct measurement of steroids is vital for the diagnosis of congenital adrenal hyperplasia (CAH), apparent mineralocorticoid excess, familial hyperaldosteronism type I, primary aldosteronism, Cushing's syndrome, adrenal insufficiency, etc. Steroid diagnostics also plays an important role in disorders of sexual differentiation and gonadal function. Steroid metabolism is involved in evaluations for precocious puberty, premature thelarche, and polycystic ovary disease. Finally, the Hypothalamo-Pituitary-Adrenal (HPA) axis is considered to be one of the major systems involved in fetal programming or in stress regulation.

Much effort has therefore been expended in order to optimize steroid diagnostics. This is true especially for adrenal gland disorders or heritable forms of hypertension, as for example, the apparent mineralocorticoid excess caused by a deficiency of 11 $\beta$ -hydroxysteroid dehydrogenase type 2.

Congenital adrenal hyperplasia (CAH) is the most common adrenal gland disorder in infants and children. Congenital adrenal hyperplasia is caused by a group of autosomal recessive disorders of adrenal cortisol biosynthesis (Forest, 2004; New and Wilson, 1999) caused by impairment of one of the enzymes required to synthesize cortisol from cholesterol in the adrenal gland (s. Figure 1). If cortisol

production is decreased, pituitary secretion of ACTH increases via the negative feedback system, which in turn causes hyperplasia of the adrenal cortex. Owing to the blocked enzymatic step, cortisol precursors accumulate in excess and are converted to potent androgens, which are secreted and cause in utero virilization of the genitalia of the affected female fetus in the classical form of CAH. Excess production of hormones proximal to the enzymatic defect gives rise to various clinical phenotypes. There is a wide spectrum of clinical presentations including a severely affected form with a defect in the aldosterone biosynthesis ("salt-wasting" type), a form with apparently normal aldosterone biosynthesis ("simple virilizing" type) and a mild "nonclassic" form that may be asymptomatic or may be associated with symptoms of androgen excess developing during childhood or at puberty. In the Caucasian population, 21-hydroxylase deficiency (Riepe and Sippell, 2007), the classical form of CAH, accounts for more than 90% of all cases, whereas 5% are caused by 11-hydroxylase deficiency. Other enzyme deficiencies and clinical phenotypes are less frequent.

In 21-hydroxylase deficiency, the conversion of 17 $\alpha$ -hydroxyprogesterone, the main substrate of the 21-hydroxylase enzyme, to 11-deoxycortisol is impaired in the pathway of cortisol synthesis (Figure 1). Aldosterone biosynthesis can be compromised because of an insufficient conversion of progesterone to 11-deoxycorticosterone. Thus, the unblocked precursors 17 $\alpha$ -hydroxyprogesterone, pregnenolone and progesterone accumulate. These steroid precursors can serve as substrates for androgen biosynthesis and are diverted in the adrenals to androgen pathways, leading to elevated levels of the androgens dehydroepiandrosterone,  $\Delta^4$ -androstenedione, and testosterone. Defective mineralocorticoid synthesis may lead to a life-threatening salt-wasting crisis. Clinical symptoms include poor feeding and failure to thrive, vomiting, hyperkalemia, hyponatremia, dehydration, metabolic acidosis, and apathy. Lifelong hormone replacement is necessary.

Screening and diagnosis of CAH relies on the specific determination of adrenal steroid precursors and the subsequent confirmation diagnostics. Because of this, much effort has been invested in order to optimizing steroid analysis for CAH.

## **1.2 Steroid analysis**

Clinically important endogenous steroids can be measured using numerous techniques, including HPLC with UV detection (Turpeinen et al., 1997), gas

chromatography (Wudy and Hartmann, 2004), and various immunoassays. So far, immunoassays are used most commonly for the monitoring of steroids. They are rapid and easy to perform and are widely used in many laboratories and hospitals because of their simplicity, speed, and sensitivity. They can be many orders of magnitude more sensitive than LC-MS/MS and can be very easily automated.

Although this technique will doubtless continue to be the method of choice for routine use in the clinical field, the reliability of steroid immunoassays has been shown to be dubious because of the lack of specificity due to interference from other endogenous steroids, lipids and to matrix effects.

Problems with steroid immunoassays have been reported for special patient groups, such as for female patients or preterm and acutely ill neonates (Herold and Fitzgerald, 2003; Taieb et al., 2003), or when analysing different media, e.g. saliva (Gröschl et al., 2001). Steroid hormone immunoassays are often not consistent, especially at normal and low concentrations (Moal et al., 2007) because of low assay specificity, inadequate standardization, and poor optimization of the methods over the large range of concentrations seen clinically (Taieb et al., 2002).

The limitation of immunoassays can also be seen from survey results for steroids such as 17-hydroxy progesterone or testosterone, which exhibit considerable lack of standardization.

Since interfering substances and the degree of interference vary among commercially available immunoassays, reference intervals are often method-specific and are not interchangeable. As a consequence, results from different assays are often not comparable. This means that individual reference ranges are required for each immunoassay, since the hormone concentrations measured in the same sample may vary considerably depending on the kit used. In each case an extensive evaluation is necessary, and this is a formidable problem, especially in pediatric endocrinology. Patient follow-up over time or between laboratories, as well as longitudinal studies are extremely difficult. Age- and gender corrected normal ranges using standardized assays are generally lacking. Another drawback of immunoassays is the fact that a different assay is needed for each steroid and also the low dynamic range.

In recent years, liquid chromatography tandem mass spectrometry has emerged as the most accurate method for measuring small molecules (Xu et al., 2007). LC-

MS/MS usually offers high specificity, even in complex sample matrices, because of the selection of a specific precursor ion in the first mass analyzer, and selection of a specific fragment ion formed during passage of the precursor ion through a collision cell. In the last few years, specific LC-MS/MS methods for analysing steroids in different matrices have been developed. In addition to the high specificity there are several other advantages. In contrast to immunoassays, which allow the measurement of only a single steroid, LC-MS/MS has the advantage that several steroid hormones can be measured simultaneously (Holst et al., 2007). This profiling allows evaluation of the status of the enzymes involved in the pathway and thus provides a better tool for diagnosis than does the measurement of one steroid alone. LC-MS/MS is suitable for routine use and should overcome the discussed problems regarding specificity and efficiency with high throughput. In contrast to GC-MS, no complex time consuming workup and derivatisation of the samples is necessary. GC-MS methods show good specificity, but usually have low throughput and require large sample volumes. They are usually restricted to few specialized clinical laboratories (Wudy et al., 1995).

This review concentrates on the clinical applications of steroid analysis from the point of view of a pediatric endocrinologist. In the following, examples for this approach for assays in pediatric endocrinology show the great advantages and also the substantial technical progress that has been achieved with the introduction of LC-MS/MS for clinical steroid analysis.

## **2. Methodological aspects of LC-MS/MS**

A number of LC-MS/MS-based methods using different ion sources have been reported for the determination of different steroid hormones. In Table 1 an overview of different assays, with references, are given for the following steroids: aldosterone, androstenedione, corticosterone, dehydrocorticosterone, cortisol, cortisone, dehydrocorticosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-3-sulfate (DHEAS), deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dihydrotestosterone, 17 $\alpha$ -hydroxyprogesterone, 17hydroxypregnenolone, estradiol, estriol, pregnenolone, progesterone, and testosterone. The main methodological characteristics of the LC-MS/MS measurement procedures are summarized. In the following, important methodological aspects referring to ionization mode, sample



preparation and chromatography are discussed in brief. For methodological details, the reader is referred to different reviews (Xu et al., 2007) and corresponding publications .

The LC-MS/MS-based methods mentioned above use different ionization sources (see. Table 1): There are three optional modes, namely electrospray ionization (ESI), heated nebulizer [atmospheric pressure chemical ionization (APCI)] and atmospheric pressure photoionization (APPI). The electrospray ionization source is considered more sensitive than the APCI source for polar compounds. However, for the nonpolar or low-polar compounds, which comprise most steroid molecules, the sensitivity provided by the electrospray ionization source is often less satisfactory than the APCI source. The more recently introduced APPI source has been demonstrated as significantly more sensitive than APCI for certain compounds (Guo 2004).

Most steroids are measured in the positive mode. Some, such as aldosterone, were analyzed in the negative mode (Guo et al., 2006; Turpeinen et al., 2008). Despite structural similarities among steroids, the efficiency of soft ionization methods for their detection is very different. The ionization efficiency of each steroid must be studied and can be different using different ion sources and instruments (Janzen et al., 2008; Kushnir et al., 2004; Kushnir et al., 2006b; Vogeser et al., 2001a). Kushnir, for example, observed the highest absolute intensity for 17-hydroxyprogesterone with APPI followed by APCI and ESI. APPI is much less prone to ion suppression than either APCI or ESI. Evidence indicates that ESI is especially susceptible to ion suppression effects. The high linear dynamic range and the applicability to a broad range of polarity makes APCI a good choice for steroids, especially when the APPI source is not available.

The physiological endogenous concentrations for several steroids are very low , and the sensitivity of tandem mass spectrometry is not adequate. Derivatization can enhance the ionization efficiencies, leading to higher sensitivity and specific detection (Higashi and Shimada, 2004). Improvements in sensitivity of detection could be achieved by the formation of oxime or picolinoyl derivatives, and by optimization of the sample preparation and ionization (Kushnir et al., 2006b; Kushnir et al., 2008; Yamashita et al., 2007).

Chromatographic separation plays an important role in the method performance. The analytical column must provide sufficient retention and separation of the steroids. Most fragment ions observed in their tandem mass spectra are common to different components (Williams et al., 1999), and a complete specificity is therefore not possible (see Table 1, for example corticosterone, 11-deoxycortisol and 21 deoxycortisol or aldosterone and cortison). Steroid compounds tend to lose water molecules easily in the source, which further reduces the selectivity. Moreover, as fragments are common to several steroid compounds, the selectivity will not be as high as required. As a consequence, a good chromatographic separation is indispensable to support the MS/MS method. This is especially important in the low concentration range. Therefore, detailed interference studies are necessary. An example is shown in Figure 2. testosterone and epitestosterone are isomers with the same molecular weight, they cannot be differentiated by MS/MS alone and must be resolved by HPLC (Rauh et al., 2006).

Normally, chromatographic separation is carried out on a reverse phase C-18 or C8 analytic column. Monolithic columns can improve throughput since they exhibit higher separation efficiency at high flow velocities compared to conventional LC columns. Sample throughput can also be increased by the use of the new sub-2  $\mu\text{m}$  particle columns. Narrow chromatographic peaks are realized in significantly shorter chromatographic run time. However, these columns make high demands on the HPLC system (pressure up to 1000 bar) and sample preparation. As seen in Table 1, chromatographic runtimes lower than 10 min should be attained and are requisite for high throughput analysis.

To assess selectivity of the analysis in LC-MS/MS, it is advantageous to use multiple mass transitions for the analysis of interest. The use of deuterated or  $^{13}\text{C}$  labelled internal standards is highly recommended to ensure optimal accuracy. These internal standards appear to be less affected by differences in the ionization of steroids and its internal standard caused by the variability of interpatient matrices ((Guo et al., 2004; O'Halloran and Ilett, 2008). Moreover, loss resulting from extraction procedures are corrected. For example, the recovery of DHEAS was significantly increased by the use of DHEAS-d2 as internal standard (Guo et al., 2004; Guo et al., 2006). The stability of the deuterium label should be examined for the specific experimental conditions to avoid exchange reactions.

In addition to the chromatography, the sample preparation is also a critical aspect. Different techniques are used (see Table 1), such as liquid-liquid extraction (Moal et al., 2007) and solid phase extraction (Herold and Fitzgerald, 2003), normally with a protein precipitation step beforehand. The aim in a routine clinical laboratory should be rapid pre-analytical sample processing with minimal manual sample manipulation, providing the desired sensitivity and high specificity. The on-line SPE technique offers speed, high sensitivity due to the preconcentration factor, and low extraction cost per sample, but generally requires the use of program controlled switch valves and column re-configurations. Several generic approaches have been developed for on-line extraction coupled to LC-MS/MS (Rauh et al., 2006; Vogeser et al., 2001a). In contrast liquid liquid extraction requires no additional equipment, but automatisaion is difficult. Sample preparation must be optimized for each individual analyte. Labor intense manual steps should be avoided.

As well as the measurement of the free steroids, LC-MS/MS can also be deployed for the analysis of steroid conjugates (sulphates or glucuronides). Higashi et al. (Higashi et al., 2007) developed a simple and sensitive LC-MS/MS method for the simultaneous quantification in saliva of representative 17-ketosteroid sulphates, dehydroepiandrosterone sulphate (DHEAS), androsterone sulphate and epiandrosterone sulphate. These sulphated conjugates are also present at relatively high concentrations in humans and the measurement of their levels is useful for the elucidation of individual androgen status. A method based on LC-MS/MS for the direct quantification of glucuronides in urine has been developed by Pozo et al. (Pozo et al., 2008). LC-MS/MS allows for the direct determination of glucuronides of testosterone, epitestosterone, androsterone, and etiocholanolone avoiding hydrolysis and derivatization, which are usual steps in commonly used methods based on gas chromatography-mass spectrometry (GC-MS).

The possible widespread use of LC-MS/MS is in conflict with the high cost of the instrument. LC-MS/MS systems are still expensive analytical tools. An estimation of the costs has been made by Vogeser and Seger. (Vogeser and Seger, 2008). Their calculation shows that the assay costs are comparable to high volume immunoassay tests such as TSH if a high sample throughput is provided. The costs per single analyte is decreased all the more by performing multiparametric analyses. LC-MS/MS is most cost effective when it is also used for other purposes and

compounds. Nevertheless, the use of a LC-MS/MS instrument requires specialized technical experience and is still restricted to a rather small number of clinical laboratories.

### **3. Applications of LC-MS/MS**

In the following, applications of LC-MS/MS assays for different steroids are discussed. The focus is on the most important characteristics of LC-MS/MS: the high increase of specificity using LC-MS/MS, the possibility of multi-target analyses for the elucidation of disease mechanisms, and the increase of diagnostic specificity, the good practicability of LC-MS/MS for enzyme activity assays and the high flexibility when using other specimens, for example saliva and hair (Kaplowitz and Soldin, 2007).

#### **3.1 17-Hydroxyprogesterone**

As discussed before, 17-hydroxyprogesterone is important for the diagnosis and monitoring of hyperandrogenic disorders, most importantly 21-hydroxylase deficiency. Cross reactivity, limited reproducibility and comparability are well recognized problems of 17-hydroxyprogesterone immunoassays. The lack of specificity is particularly unfortunate in the newborn period and early infancy. LC-MS/MS methods have been developed for quantification (Etter et al., 2006; Kushnir et al., 2006b; Rauh et al., 2006; Turpeinen et al., 2005; Wudy et al., 2000). The discrepancy between the LC-MS/MS method and the immunoassay is highly variable. Depending on the patient group measured, we find a tremendous discrepancy in the values (Rauh et al., 2006). Other steroids of similar structure and abundance in the circulation cause assay interference, especially in the newborn period. Although immunoassays use liquid-liquid extraction to eliminate interfering compounds, these methods are still susceptible to interferences from other endogenous steroids (Ismail et al., 2002). Extensive and laborious sample pretreatment lowers the overall efficiency and makes these methods awkward for practical use in a clinical laboratory. Moreover, LC-MS/MS provides more accurate results than RIA and makes analysis of serum/plasma samples possible where interferences are likely to cause false high results.

Another example is 17-hydroxyprogesterone in saliva. Salivary analysis is a proven alternative to plasma for the determination of hormones (Gröschl et al., 2003; Higashi

et al., 2007; Lewis, 2006). The non-invasive collection is convenient for diagnosis in children, or for out-patient sampling. Depending on which specific hormones are quantified in salivary diagnostic assays, the collection device itself may influence the accuracy of testing. Care must be taken to select a collection device for salivary diagnosis which is appropriate for the assessment the particular steroid hormone required (Gröschl and Rauh, 2006).

Excellent correlation is reported for 17-hydroxyprogesterone (Walker et al., 1979). Currently, the standard medical treatment of CAH consists of giving a glucocorticoid (a cortisol-like steroid) medication, e.g., oral hydrocortisone in children, prednisone or dexamethasone (dexamethasone in older patients) (Forest, 2004).

The first goal of treatment for CAH is to bring the patient's androgen levels back to normal. The diurnal variation of plasma 17-hydroxyprogesterone is reflected by similar changes in salivary 17-hydroxyprogesterone and hence timed salivary 17-hydroxyprogesterone has been used for diagnosis. Early morning salivary 17-hydroxyprogesterone is useful as a screening tool for adrenal suppression and salivary 17-hydroxyprogesterone is helpful in monitoring the hormone replacement therapy and patient compliance (Figure 3).

Normally treatment of CAH is controlled on the basis of urinary steroid profiling (Caulfield et al., 2002) which has the advantage of being independent of most steroid hormone circadian rhythms. The main metabolite of 17-hydroxyprogesterone in urine is pregnanetriol which reflects the 17-hydroxyprogesterone levels over time. The excretion of pregnanetriol was measured by GC-MS. If we compare the pregnanetriol excretion with salivary 17-hydroxyprogesterone (s. Figure 3), we see an excellent correlation. This emphasises the usefulness of salivary steroid measurements. The great advantage using of mass spectrometry is that we can use the same method for plasma and saliva. No matrix effects or cross reactivity need be considered.

### **3.2 Testosterone**

For children, the circulating testosterone concentration is determined principally for the diagnosis, treatment, and gender assignment of newborns or young infants with ambiguous genitalia. The testosterone concentration is also used to determine pubertal stage in association with physical examination and gonadotropin determination, and in the follow-up of children with precocious or delayed puberty. In all of these situations, a highly sensitive and very accurate testosterone assay is

required. The measurement of testosterone, as done in most laboratories, suffers from a number of serious problems. Automated analyzers are easy to use, have short cycle times, and have become established in many clinical laboratories. While these assays may be valid in adult men, given their higher levels of hormone, these methods do not produce accurate results in children or females with lower levels of these steroids. Taieb et al (Taieb et al., 2003) show that none of the immunoassays tested was sufficiently reliable enough for the investigation of the low and very low testosterone concentrations expected in sera from children and women. These assays are therefore unlikely to be useful for diagnosis, follow-up of sexual differentiation, or for general use in pediatric surveys.

The importance of this issue is highlighted by a position statement of the Endocrine Society (Rosner et al., 2007) which demands the improvement of accuracy and precision of testosterone tests. Several LC-MS/MS methods have been described for the quantification of testosterone in serum/plasma to overcome this problem (Cawood et al., 2005; Ismail et al., 2002; Kushnir et al., 2006a; Moal et al., 2007; Rauh et al., 2006; Singh, 2008; Taieb et al., 2003).

The immunoassays in our routine clinical steroid analysis have been replaced by a LC-MS/MS method for the simultaneous measurement of 17-hydroxyprogesterone, testosterone and androstenedione (Rauh et al., 2006). The LC-MS/MS procedure is based on an online extraction and allows measurement of endogenous concentrations of adrenal steroids in all age groups for females and males. With an instrumental analysis time of 6 min, the assay is a good alternative to immunoassays. The small sample volume (100  $\mu$ l) makes the method particularly useful for pediatric testing. Within run (CV < 15% except at the detection limit) and total coefficient (CV < 15%) were both acceptable. The lowest limit of detection is about 0.03 – 0.06  $\mu$ g/. This is still sufficient for monitoring steroid concentration in pediatric samples.

### **3.3 Cortisol/cortison**

Epidemiological studies (Fowden and Forhead, 2004) in diverse populations have demonstrated a link between low birthweight and disease in later life. Various studies have confirmed this association between low birthweight and an increased risk of cardiovascular and metabolic disorders, including hypertension and insulin resistance in adult life (Ibanez et al., 2006; Levy-Marchal and Czernichow, 2006; Plank et al.,

2007b). This process is known as 'foetal programming of adult disease', but the underlying mechanisms are not yet clear.

It has been suggested that 11  $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) may be involved (Struwe et al., 2007). Placental 11 $\beta$ -HSD2 builds up a physiological barrier for the foeto-placental unit in order to protect the foetus from glucocorticoid excess. Increased glucocorticoid exposure of the foetus leads to intrauterine growth retardation (IUGR) and an increased predisposition to the development of type II diabetes or hypertension in later life (Stocker et al., 2005). Furthermore a persistence of the hypothalamus -pituitary -adrenal axis dysregulation into postnatal life is assumed to play an important role in the pathogenesis of hypertension. The changes in the activity of 11  $\beta$ -hydroxysteroid dehydrogenase type 2 (11  $\beta$ -HSD2) and the ensuing disequilibrium between cortisol and cortisone are thought to be a key mechanism for the formation of hypertension in later life in these patients (Hershkovitz et al., 2007; Ojeda et al., 2008).

LC-MS/MS enables the simultaneous determination of cortisol and cortisone with highest specificity and precision from a single serum sample (Kushnir et al., 2004; Tenhola et al., 2005; Vogeser et al., 2001b). This allows the determination of cortisol cortisone ratios as a marker for 11 $\beta$ -HSD activity within a short turn-around time. In a cross-sectional, retrospective study, Plank et al. (Plank et al., 2007a) investigated whether prenatal programming leads to alterations in cortisone/cortisol ratios on a systemic level. They analysed sera of 132 children born small for gestational age (SGA) (aged 2–13 years) with persistent short stature [ $< -2$  standard deviation score (SDS)] and of 25 children born appropriate for gestational age (AGA) (aged 4–11 years) with normal body height. Cortisol, cortisone and the cortisone/cortisol ratio in serum did not differ in these children when comparing SGA to those born AGA with normal height. The concentrations were independent of weight and length SDS at birth, as well as of gestational age.

Another application is the follow up of changes of the serum cortisone cortisol ratio caused by surgical stress and systemic inflammation, and the diagnosis of 11 $\beta$ -hydroxy-steroid-dehydrogenase deficiency in hypertension research (Vogeser et al., 2003).

Salivary cortisol determinations have proved popular in psychobiology, stress and sports medicine studies (Gröschl et al., 2002; Kirschbaum and Hellhammer, 1989). The prime advantage of saliva is that it offers non-invasive, stress-free and real-time

repeated sampling where blood collection is either undesirable or difficult. It is well suited for pediatric, time-shift and psychobiological studies. In addition, no special training or equipment is needed and subjects can collect samples conveniently themselves, if required. Salivary steroid levels may reflect the circulating level of free steroid, rather than total circulating levels, which may be disturbed by the presence of circulating high-affinity-binding proteins. Their use is based on the assumption that salivary cortisol is a reasonable reflection of hypothalamic-pituitary-adrenal axis function. Indeed, in the diagnostic setting, salivary cortisol levels parallel those in plasma following ACTH and CRH stimulation and following exercise-induced stress (Pruessner et al., 1997). However, the correlation of salivary cortisol levels with total plasma cortisol is hampered by the presence of corticosteroid-binding globulin which is saturated at a concentration of 500-600 nmol/L of cortisol. There is evidence that saliva levels reflect the unbound concentrations in plasma (Umeda et al., 1981). This is shown in Figure 4 for children before and after hemodialysis therapy. The diagnostic value of salivary cortisol in end stage renal disease was shown by Arregger et al. (Arregger et al., 2008). Recently, there has been considerable interest in the use of night time salivary cortisol for the initial screening of Cushing's syndrome.

The salivary gland has abundant 11- $\beta$ -hydroxysteroid dehydrogenase type 2 activity and, as a consequence, saliva, unlike plasma, has up to five times the level of cortisone compared to cortisol. Unfortunately, reported cut-off values differ considerably. Depending on the relative cross-reactivity of cortisol antibodies towards cortisone, there could be quite variable values for salivary cortisol measured by different immunoassays (Baid et al., 2007). Measurement by LC-MS/MS can overcome this problem.

### **3.4 Assay of steroid converting enzymes**

LC-MS/MS methods can also be used for the very sensitive assay of steroid-converting enzyme activity in tissues (Miksik et al., 2004). Investigations into the metabolic conversion in tissues and cells have been rather limited. One problem is the fact that such studies have to use radioactive steroid hormones to obtain sufficient sensitivity (Solly et al., 2005).

One example of a sensitive, non-radioactive LC-MS/MS method to study corticosterone metabolism in lysates of different tissues is shown in Figure 5. Using a



specific liquid chromatography-tandem mass spectrometry method for analysing corticosterone and dehydrocorticosterone concentrations (Plank et al., 2007a; Usa et al., 2007) the activity of 11 $\beta$ -HSD in different tissues is investigated. Powdered tissue samples were homogenized and microsomes were isolated. The microsomes were incubated in homogenisation buffer containing 100  $\mu$ g/L corticosterone and 100 mM NAD for 75 min at 37°C. The diagram (Figure 5) shows the results of this experiment. As expected, high activity was found in kidney and lung. The method is suitable for high throughput. It is sensitive, robust and is useful for the quantification of types 1 and 2 activity.

### 3.4 Steroid profiling

Steroid profiles (Guo et al., 2004; Guo et al., 2006; Guo et al., 2008; Janzen et al., 2008; Kao et al., 2001; Kushnir et al., 2006b) allow for the acquisition of more clinically useful data than can be obtained through the measurement of a single steroid. The spectrum of diagnostically important metabolites in a plasma steroid profile is particularly helpful for a correct diagnosis and is also useful for the differential diagnosis of nonclassical forms of CAH, which may be indistinguishable using clinical presentation and sonography alone. (Holst et al., 2007; Sahin and Kelestimur, 1997)). Furthermore, a steroid profile contains all steroid metabolites necessary for the diagnosis and monitoring of female hyperandrogenism and is useful for monitoring treatment and compliance in CAH due to enzyme deficiencies, and after adrenal or gonadal tumor surgery.

In Figure 6 three examples of such steroid profiles are shown: a reference sample, a CAH patient during treatment and a patient with 17-hydroxylase deficiency. The steroid profile of the patient with 17-hydroxylase deficiency is dominated by corticosterone and dehydro-corticosterone lacking cortisol and cortisone. The patient with 21-hydroxylase deficiency had a highly increased serum 17-hydroxyprogesterone and 21-deoxycortisol (nondetectable in sera of healthy subjects).

In the pediatric setting, it is highly advantageous that only small amounts of plasma are needed to enable the determination of a whole profile of diagnostically important steroid hormones at the highest specificity. Guo et al. (Guo et al., 2006) described a second generation steroid profile assay which allows for the quantitation of 12

steroids (aldosterone, androstenedione, cortisol, corticosterone, 11-deoxycortisol, estradiol, DHEAS, DHEA, 17-hydroxyprogesterone, 25-hydroxyvitamin D<sub>3</sub>, progesterone, testosterone) simultaneously in 11 min, using an API 5000 coupled with the APPI source. This method requires a sample volume of 200 µl.

Steroid profiling is also very useful for neonatal screening for CAH in order to reduce the high number of false-positive results produced by immunoassays (Marsden and Larson, 2004). Neonatal screening for CAH was first introduced in 1977, when immunoassays became available. Since then, most screening laboratories have used these tests which, unfortunately, produce a large number of false-positive results. Preterm babies often show high levels of 17-hydroxyprogesterone because of stress or the delayed maturation of 11-hydroxylase. Furthermore, cross-reactions of the specific antibody with other steroids contribute to the high number of false positive results. Compared with other neonatal screening tests, the specificity of screening for 21-hydroxylase deficiency by immunoassay is low.

Several groups have developed steroid profiles in order to minimize false-positive results attributable to cross-reactivity (Lacey et al., 2004). For example, the group of Sander (Janzen et al., 2008) applied a method which allows rapid quantitative analysis of the steroids 21-deoxy-cortisol, and 17-hydroxy-progesterone, and of cortisol as downstream product. A sensitive ratio can be used to distinguish between true 21-hydroxylase deficiency and false – positive results. Most importantly, the steroid profile includes 21-Deoxycortisol, the most relevant marker for 21-hydroxylase deficiency. The LC-MS/MS method allows immediate confirmation of a 21-hydroxylase deficiency, since only a small amount of material is needed which can be taken from the original filter card.

## Conclusions

Tandem mass spectrometry usually offers high specificity, even for complex sample matrices. It can simultaneously measure several steroids in serum/plasma and other matrices with minimal sample preparation. It can be employed routinely in a clinical environment and is attractive because of the simplicity of sample processing and high throughput. Steroid profiles provide additional information concerning adrenal function. LC-MS/MS allows for simple, accurate, and simultaneous measurement of multiple steroids in large numbers of samples, and improves the diagnostic accuracy.

In addition to the high throughput, the assay requires minimal sample preparation and requires a small volume of serum/plasma. All these features make it an attractive assay to use in a clinical setting. LC-MS/MS seems to have the potential to overcome the limitations of immunoassays. The future will show whether metabolic profiling approaches will change diagnostic strategies and whether multiparametric assays will be introduced into practical medicine.

However the widespread application of this technology requires substantial further development until it can be used as a routine instrument in the setting of clinical laboratories (Vogeser and Seger, 2008). High-level reference method protocols should be developed through inter-laboratory collaboration (Thienpont et al., 2008). Standardization is necessary relating to calibration and validation parameters to establish normative values for steroids in children. This would be facilitated if commercial certified kits including calibrators, internal standards and quality controls for the LC-MS/MS analysis were available. Nevertheless, steroid measurement in laboratory medicine urgently needs an improvement of accuracy and LC-MS/MS has an emerging role as a powerful tool in this process.

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Figure 1. Pathways of steroid biosynthesis: (**3 $\beta$ -HSD**: 3 $\beta$ -hydroxysteroid dehydrogenase, **11 $\beta$ HSD**: 11 $\beta$ -hydroxysteroiddehydrogenase, **18-HSD**: 18-hydroxysteroiddehydrogenase, **17 $\alpha$ -H**: 17 $\alpha$ -hydroxylase, **21-H**: 21-hydroxylase, **11 $\beta$ -H**: 11 $\beta$ -hydroxylase, **18-H**: 18-hydroxylase, **20,22-D**: 20,22 desmolase, **17,20-L**: 17,20-lyase).

Figure 2. Multiple-reaction monitoring chromatograms of calibrator samples: a) epitestosterone 5  $\mu$ g/L, b) testosterone 1 $\mu$ g/L. Mass transition m/z 289/109, Liquid chromatography conditions as in Rauh et al.(Rauh et al., 2006).

Figure 3. Distribution of salivary 17-hydroxyprogesterone values and urinary pregnanetriol, obtained from a CAH patient during 53 months. Pregnanetriol was measured by GC-MS.

Figure 4. Correlation of free cortisol with salivary cortisol concentrations for children (n = 25) before and after hemodialysis therapy ( $C_{(\text{salivary cortisol})} = 0.70 * C_{(\text{free cortisol})} + 0.24$ , r = 0.95).

Figure 5. 11 $\beta$ -HSD activity assay of microsoms obtained from different rat tissues. Concentration of dehydrocorticosterone versus time ( $C_0$  (corticosterone) =100  $\mu$ g/L, 100 NAD<sup>+</sup>, 18 mg/L protein concentration, 75 min at 37°C).

Figure 6. Plasma steroid profiles of three patients: patient with normal adrenal function, patient with 21-hydroxylase deficiency, patient with 17-hydroxylase deficiency.

**Tab. 1: Method parameters of LC-MS/MS assays for different steroids**

Abbreviations : #Derivatives,\* LOQ, P: protein precipitation, L: liquid extraction, S: solid phase extraction, O: online extraction

Analyte	Mass transition	Ionization	IS	LOD (ng/L)	Chromatography	Time (min)	Sample volume (µL), preparation	Reference
Aldosterone	359/189	ESI-	D7	11*	C18 50*2.1 mm, 3.5µm	10	500, L	Turpeinen 2008
	359/331	APPI-	D6	10	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
Androstendione	287/97	APPI+	D7	1.5	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
		ESI+		403	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
	287/109	APCI+	D7	100*	Monolithic C18 100*4.6 mm	6	100, P-O	Rauh 2006
Corticosterone	347/121	ESI+	D8	100**	C18 33*4.6 mm, 3 µm	4	50, L	Usa 2007
	347/329	APPI+	D8	2	C8 33*3.0, 3 µm	11	200, P-O	Guo 2006
		ESI+		1142	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
Cortisol	363/121	APPI+	D4	1.5	C8 33*3.0, 3 µm	11	200, P-O	Guo 2006
		ESI+	D2	272	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
	363/309	APPI+	D4	1000	Phenyl/Hexyl 50*2.1mm, 5µm	3	100, L	Kushnir 2004
		ESI+	D3	1000	C18,125*2, 5µm	4.5	500, P-O	Vogeser 2001
Cortisone	361/163	APPI+		5000	Phenyl/Hexyl 50*2.1mm 5 µm	3	100, L	Kushnir 2004
		ESI+			C18,125*2mm, 5µm	4.5	500, P-O	Vogeser 2001
Dehydro-corticosterone	345/121	ESI+		100*	C18 33*4.6, 3 µm	4	50, L	Usa 2007
DHEA	271/213	APPI+	D2	10	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
DHEAS	271/213	APPI+	D2	2	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
Deoxycorticosterone	331/109	ESI+		762	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
Dihydrotestosterone	291/255	ESI+		853	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
11-Deoxycortisol	347/97	APPI+	D2	5	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
	347/109	ESI+		571	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
	377/124#	ESI+	D2	25	C18 50*2.0mm, 4 µm	7.2	200,L	Kushnir 2006b
21-Deoxycortisol	347/311	ESI+			C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
17-OH-Progesterone	331/109	APPI+	D8	1.5	C8 33*3.0mm, 3 µm	11	200, P-O	Guo 2006
		ESI+	D8	327*	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
	331/97	ESI+	D8	33	C16amide 50*4.6mm, 5µm	7	1000,S	Etter 2006
		APCI+	D8	100*	Monolithic C18 100*4.6mm	6	100, P-O	Rauh 2006
		ESI+	D8	330	C18 55*2 mm	10	500, L	Turpeinen 2005
		APCI+	D4	20	C18 125*3 mm, 5µm	12	1000, L	Wudy 2000
361/124#	ESI+	D8	25	C18 50*2.0 mm, 4 µm	7.2	200, L	Kushnir 2006b	
17-OH-Pregnenolone	348/330*	ESI+	D3	25	C18 50*2.0 mm, 4 µm	7.2	200, L	Kushnir 2006b
Estradiol	255/159	APPI+	D4	10	C8 33*3.0 mm, 3 µm	11	200,P-O	Guo2006
	271/145	ESI-	D4	2	C8 33*3.0 mm, 3 µm	8	200, P	Guo 2008
	507/156#	ESI+	D3	1*	Phenyl 100*2.0, 3 µm	8	200,L-O	Kushnir 2008
	483/264#	ESI+	<sup>13</sup> C4	1*	C18 150*3 mm, 3µm	10	1000, L-S	Yamashita 2007
Estriol	287/171	ESI-	D2	2	C8 33*3 mm, 3 µm	8	200,P	Guo 2008
Pregnenolone	332/300*	ESI+	D4	25	C18 50*2.0 mm, 4 µm	7.2	200, L	Kushnir 2006b
Progesterone	315/109	APPI+	D9	1.5	C8 33*3.0 mm, 3 µm	11	200 P-O	Guo 2006
		ESI+		1978	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
Testosterone	289/109	APPI+	D2	1.5	C8 33*3.0 mm, 3 µm	11	200 P-O	Guo 2006
		ESI+		596	C18 20*2.1mm, 3 µm	6	15, L	Janzen 2008
		APCI+	D5	100*	Monolithic C18 100*4.6 mm	6	100, P-O	Rauh 2006
	289/97	APCI+	D3	70*	C18 33*4.6 mm	7.5	100, P-O	Singh 2008
		ESI+	D5	50*	C8 150*2.1 mm 3 µm	20	500, L	Moal 2007
	304/124#	ESI+	D2	72*	C18 50*4.6 mm, 2.5 µm	4.8	50, P	Cawood 2005
		ESI+	D3	5	C18 50*2.0 mm, 5 µm	3	100, L-S	Kushnir 2006a

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