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Review

Steroid measurement with LC–MS/MS. Application examples in pediatrics☆

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ABSTRACT

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 disease, 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.

Most methods for the determination of steroid hormones are based on immunoassays, which are rapid and easy to perform. However, the reliability of several steroid immunoassays has been shown to be questionable because of the lack of specificity and of matrix effects. Immunological methods, especially direct assays, often overestimate true steroid values. Patient follow-up over time or between laboratories, as well as longitudinal studies, are therefore extremely difficult. This is of particular importance in pediatrics.

Liquid chromatography triple quadrupole mass spectrometry (LC–MS/MS) is an increasingly common tool in the clinical laboratory and has the potential to overcome the limitations of immunoassays. LC–MS/MS affords the specificity, imprecision, and limits of quantification necessary for the reliable measurement of steroids, expanding diagnostic capabilities. In addition to the high throughput, the method requires minimal sample preparation and a small sample volume. All these features make it an attractive method to use in a clinical setting.

Moreover, LC–MS/MS has the advantage that a spectrum of steroid hormones can be measured simultaneously. 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. Steroid profiles open up new vistas. The applicability for clinical samples and questions in pediatric endocrinology will be discussed.

Contents

☆ Article from special issue on "Steroid profiling and analytics: going towards Sterome".

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1. Introduction

Steroid assays play an important role in the clinical evaluation of a number of common endocrine disorders [1]. Much effort has therefore been expended in order to optimize steroid diagnostics [2,3].

One significant milestone for the diagnosis and clinical investigations of normal physiology and pathophysiology was the development of radioimmunoassays [4]. Extraction and column or paper chromatography followed by radioimmunoassay enabled enough sensitivity and specificity. Labor intensity, cost, and turnaround time made this assays awkward for clinical routine laboratories. As a consequence kits without extraction steps and kits using a platform system were developed. So far, the most common assays used for the monitoring of steroids are immunoassays. They are rapid and easy to perform and are widely used in many laboratories and hospitals because of their simplicity, speed, and sensitivity. These methods have been proven to be cost-effective, but they have limitations. Since the advent of high throughput, direct assays on automated analyzers, the reliability of many steroid immunoassays has been shown to be questionable because of the lack of specificity and matrix effects [5]. In exchange for high volume and high throughput handling of many specimens in a short period of time, automated testing generates results that are sometimes far less sensitive than the original radioimmunoassay methods. Another drawback of immunoassays is the fact that you need a different assay for each steroid. Except for luminescence assays, the dynamic range is low.

In the same time frame GC and GC–MS assays were developed [6]. GC bears the greatest potential for separating steroids, and mass spectrometry (MS) allows for the highest specificity in determining steroid metabolites. But, apart from a few specialist laboratories, its use is restricted to the characterization of steroids, especially in research. Quantitation by GC–MS was mostly not suitable for routine clinical use because of high labor-intensiveness, cost, and poor sensitivity.

In recent years, liquid chromatography tandem mass spectrometry is becoming an increasingly important analytical technology in the clinical laboratory environment [7,8]. One of the first applications was multiple analyte screening for inborn errors of metabolism [9]. Another major stimulus for the introduction has been the appearance of new immunosuppressive drugs and the development of multiple-drug therapeutic regimens [10,11]. So, LC–MS/MS is suitable for routine use and should overcome the discussed problems regarding specificity and efficiency. In the following, the focus will be on special aspects of pediatric endocrinology, which show the enhancing diagnostic capabilities of LC–MS/MS.

2. LC–MS/MS

In recent years, liquid chromatography tandem mass spectrometry has emerged as the most accurate method for measuring small molecules [12,13]. The majority of the LC–MS/MS methods for quantitative steroid analysis use triple quadrupole mass spectrometry. MS/MS is mostly performed in multiple-reaction monitoring (MRM) mode which is highly selective and sensitive. Both mass analyzers are fixed on transmission of the compound-specific precursor and product ions reducing chemical noise and enhancing signal-to-noise ratio. In combination with LC this technique has a very high analytical specificity and sensitivity.

Ionization techniques commonly used for the analysis of steroids with LC–MS/MS include atmospheric pressure chemical ionization (APCI), photo ionization (APPI) [14], and electrospray ionization (ESI). The type of ionization preferred in the different assays depends on the chemical properties of the analyte (presence of ionized or ionisable groups, polarity) and instrument characteristics. Generally, ESI is preferred for molecules with high polarity, whereas APCI and APPI are used for molecules with low to medium polarity. Most steroids are measured in the positive mode. Some, such as aldosterone are analyzed in the negative mode [15]. In some applications chemical derivatization is used to gain sensitivity. Derivatization can change the efficiency of the ionization, fragmentation, chromatographic retention, and matrix effects [16].

LC–MS/MS has a number of advantages. Beside the high specificity, sensitivity and throughput, sample extraction and pretreatment are minimized. In contrast to GC–MS, no complex, time consuming workup and derivatisation of the samples is necessary [17]. Sample clean up can be reduced and analysis time can be shortened. In contrast to immunoassays, one main advantage is the possibility to measure many steroid hormones very specifically in parallel within one analysis. Furthermore, the dynamic range of the calibration spans four orders of magnitude instead of two as for EIA or RIA. Because of these characteristics, LC–MS/MS is a well suited technique for the analysis of steroid hormones.

However, all methods have to be extensively validated to assure their accuracy and the diagnostic utility. The evaluation of matrix effects on the quantitative analysis is an important and sometimes overlooked aspect of assay validation. To date, the instrumentation is more complex than the typical analyzer, and the use of a LC–MS/MS instrument requires special technical experience.

For methodological details, the reader is referred to different reviews [7,12] and corresponding publications [16,18,19]. For more general reviews of LC–MS/MS, see Ref. [20–22].

3. Special challenges in pediatrics

Challenges in measurement of steroids in the blood of children are related to low physiologic concentrations and the presence of endogenous compounds that can interfere. In addition there is a special challenge in interpretation. Examples of misleading values of steroids illustrate the added expense and difficulty reaching a diagnosis among children, together with additional complexity of the medical assessment and stress for parents already having an infant with significant problems [23].

Such an example involves a male newborn born at 33-week to healthy parents. A blood sample for newborn screening was taken after birth. 17-OH-progesterone was elevated using time-resolved fluorescence immunoassay (124 nmol/l; cut off <80 nmol/l). The 17-OH-progesterone measurement was repeated. The baby showed elevated 17-OH-progesterone levels on all three consecutive samples. The suspected diagnosis: congenital adrenal hyperplasia (CAH). The child had to stay at the hospital. Reevaluation at day 30 by another external laboratory resulted in further increased plasma 17-OH-progesterone concentration. After four weeks the child was transferred to another hospital. Here 17-OH-progesterone was measured by LC–MS/MS: The value was in the normal range. Genetic testing confirmed the diagnosis: No CAH. The child could be discharged from the hospital. The initial high value may have been a consequence of cross-reacting steroids in a neonate. A specific assay would have avoided this problem. One month fear and uncertainty for the family could have been prevented.

Interestingly there was always a close relationship between pediatric endocrinology and laboratories spearheading techniques of steroid analysis. What is the reason for this alliance and what are the special challenges in pediatrics?

3.1. Challenges in specificity

The accurate and precise measurement of steroids has challenged laboratorians for years since immunoassays methods for

steroids have been established. The issue of poor clinical correlation to steroid test results has been subject for discussion and concern for some time by endocrinologists. Especially in pediatrics the limitations of the assays are apparent. In children, the lack of accuracy and sensitivity of most of the immunoassays has resulted in severely limited utility as was shown for testosterone, estradiol, and 17-hydroxyprogesterone. The lack of specificity is of particular importance in the newborn period and early infancy.

The measurement of testosterone, as done in most laboratories, suffers from a number of serious problems [24,25]. Proficiency test results have demonstrated considerable variability among the many automated instruments. A great lack of standardization has been observed. The importance of this issue is highlighted by a position statement of the endocrine society [26] which demands the improvement of accuracy and precision of the tests especially testosterone. While these assays are validated in adult men, given their higher levels of hormones, these methods do not often produce accurate results in children or females with lower levels of these steroids.

This is true also for most of the direct assays for estradiol performed on automated platforms which suffer the same degrees of inaccuracy and imprecision that immunoassays do [27]. Most direct methods overestimate levels, are matrix sensitive, are influenced by the circulating level of binding globulins and cannot be used to measure accurately estradiol in children [5].

Problems with 17-hydroxyprogesterone immunoassays have been reported for newborns or when analysing different media, as for example saliva [28]. As shown by Rauh [29] and Wudy [30], the discrepancy between the MS method and the immunoassay varies. Dependent on the patient group measured they find a tremendous discrepancy of the values. Other steroids of similar structure and abundance in the circulation lead to assay interference especially in the newborn period. Even immunoassays which use liquid–liquid extraction to eliminate interfering compounds are still susceptible to interferences from other endogenous steroids. But, the extensive and laborious sample pre-treatment lowers the overall efficiency and makes these methods awkward for practical use in a clinical laboratory.

Cushing syndrome is a difficult diagnosis to establish. It is rare in childhood, and the symptoms may vary, but should be considered in any child with weight gain and growth failure. Examinations of the circadian rhythm of cortisol secretion and 24-h UFC (urinary free cortisol) estimations are useful preliminary investigations prior to provocative tests. UFC measurements have the advantage of providing an integrated measure of cortisol secretion. However, many assays lack specificity, with large number of urinary metabolites exhibiting significant cross-reactivity [31].

LC–MS/MS methods are among the most successful approaches to improve specificity problems inherent in many immunoassays (references see Section 4.1). Mass spectrometry provides results with much improved specificity for several steroids [16,18,19,32]. The variability of testosterone measurement results among mass spectrometry assays is substantially smaller than that reported for immunoassays [33]. Standardizing assays is required to further reduce the variability of measurement results. Despite enhanced specificity, LC–MS/MS methods are not totally free from interference. Assays should be thoroughly validated and should use strategies for the assessment of the specificity of analysis in every sample. Ion ratios or secondary ions should be used for identifying the occurrence of interference in patient samples [16,34].

3.2. Challenges in sensitivity

The accurate measurement of the low levels of testosterone and estradiol seen in normal children and in children with disorders of puberty and sexual development is critical for appropriate diagnosis and treatment. The current sensitivity is frequently inadequate to accurately diagnose pediatric disorders such as genital ambiguity or hypogonadropic hypogonadism [23,35]. Medical decision making in numerous pediatric endocrinology clinical situations may require measurement of sex steroid concentrations 100-fold lower than those in adults, often lying outside the validated ranges of many assays. In order to optimize clinical diagnosis, monitor therapy and conduct meaningful clinical research concerning normal and abnormal development among children, more accurate and reproducible values are mandatory.

In most testosterone assays the sensitivity is not adequate to differentiate prepubertal and pubertal secretion. With a sufficiently reliable assay, the range of levels for prepubertal and pubertal males could be established to differentiate these states. A low testosterone will indicate either impaired synthesis or testicular dysgenesis, whereas a normal or elevated concentration will indicate a defect of peripheral androgen action. The measurement of basal testosterone is also of value in the investigation of hypogonadism in late adolescence when subnormal concentrations indicate a need for further investigations. In other situations basal gonadal steroids of pediatric patients may be undectable, and further investigations require hCG stimulation.

Since the level of circulating estradiol in the prepubertal child is considerably less than testosterone, the issues of sensitivity being able to obtain a clinically useful value are even greater. The measurement of serum estradiol can facilitate the diagnosis of many pediatric endocrine disorders, including central precocious puberty and premature thelarche [35]. Increasing evidence points at an important function of low concentration of estradiol in prepubertal boys and girls. Unfortunately, current conventional estradiol assays are often insufficiently sensitive to enable the use of basal estradiol to detect failure of the ovary to produce the hormone in childhood. There is also no good or reliable dynamic test comparable to the hCG stimulation test for testicular endocrine function in boys.

Investigations of the physiological relevance of estradiol in children or the potential effect of exposure to endocrine disrupters with estrogenic or antiandrogenic activity on pubertal development demand for new and improved methods of analysis for accurate and sensitive evaluation of low concentrations of estradiol. New assays for estrogen should be sensitive enough to differentiate between boys and girls in prepuberty, between prepuberty and puberty in girls, and between normal girls and those with premature thelarche, who secrete only a small amount of estrogen above age-matched controls [35]. Kushnir et al. developed a high sensitivity LC–MS/MS assay for simultaneous measurement of estrone and estradiol. The authors use dansyl chloride derivatization to enhance detection. Total imprecision for the method was less than 11%. The limit of quantitation was 1 pg/ml. Their imprecision values at concentrations between 1 and 6 pg/ml confirm adequate functional sensitivity of the method at concentrations characteristic for prepubertal children. The assay requires only 200 μl of serum. Advances in mass spectrometry will achieve lower detection limits with greater precision and will be able to avoid derivatization approaches.

3.3. Challenges in interpretation

Accurate endocrine diagnosis depends on the measurement accuracy of the analytical application and appropriate interpretation of the test results in clinical context. Clinicians often deal with multiple laboratories with different levels of testing or different methods. It is of considerable importance to know what procedure one's laboratory is using and what interferences could occur, because laboratories may have different normal values, and most central hospitals and commercial laboratories are designed primarily to serve adult, rather than pediatric, patients. All immunoassays

have some degree of cross-reactivity with other steroids. The total overestimation by interfering substances is concentration dependent and there is a significant lack of agreement between the results of different immunoassays for the measurement. Different assay results are often not comparable. Matrix and steroid composition varies with age. For example, some cortisol immunoassays detect cortisol and cortisone. As the newborn's plasma contains mainly cortisone rather than cortisol during the first days of life, comparison of newborn data obtained by HPLC with published standards obtained by immunoassays may incorrectly suggest adrenal insufficiency.

The limitations of the assays have important implications for the use of these assays in pediatrics. Patient follow-up over time or between laboratories, as well as longitudinal studies, are extremely difficult [13]. Laboratories may use the range quoted by a manufacturer if using a commercial kit, but manufacturers usually state that the reference range data are provided only as a guide. 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. Age and gender corrected normal ranges, using a standardized assay are generally lacking which is a great problem, especially in pediatric endocrinology. Ideally, a range determined by analyzing samples using the same laboratory assay should be established in a population clearly defined by sex, age range, and time of day. In addition, the investigation of some diseases requires dynamic tests, and reference ranges for the response are also required.

Validated reference ranges for children poses a challenge [36]. In each case extensive evaluation is required. Because the onset and progression of puberty are so variable, additionally the Tanner stage has to be considered, which describes the onset and progression of pubertal changes. Interpretation of results is further complicated by physiological variables. Some hormones demonstrate a marked circadian rhythm, which may develop during puberty [37]. Many hormones are increased during stress, which may confuse the interpretation of a result. With the notable exception of DHEAS most adrenal steroids exhibit a diurnal variation based on diurnal rhythm of ACTH. Because the stress of illness or hospitalization can increase adrenal steroid secretion and because diurnal rhythms may not be well established in children under 3 years of age, it is best to obtain two or more samples for the measurement of any steroid.

Validated and well-characterized mass spectrometry-based methods could improve interlaboratory comparability. Requirements are standardization and harmonization of mass spectrometry-based assays, as well as the availability of commercial calibration materials. If suitable standardization is achieved, site specific reference ranges will be no longer necessary. This translates to a quality advantage, leading to better patient care. To date, pediatric reference intervals using isotope-dilution LC–MS/MS methods were published for aldosterone [36], 17OH-progesterone [29,36], dehydroepiandrosterone [36], 25-OH-vitamin D3 [36], testosterone [36,38–40] and estradiol [41]. Furthermore, reference intervals for the adrenal steroids 11-deoxycortisol, 17-OH-progesterone, 17-OH-pregnenolone and pregnenolone for males and females of different Tanner stages and age groups were established by Kushnir [42] and Meikle [40].

4. Applications of LC–MS/MS

4.1. Steroid assays

A number of LC–MS-based methods using different ion sources have been reported for the determination of steroids: aldosterone [15,43], androstenedione [10,15,29,44], corticosterone [15,44,45], dehydrocorticosterone [45], cortisol [10,15,44,46,47], cortisone [46,47], dehydrocorticosterone [45], dehydroepiandrosterone (DHEA) dehydroepiandrosterone-3-sulfate (DHEAS) [15,48], deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol [44], dihydrotestosterone [25,49], 17-hydroxyprogesterone [15,29,44,48,50], 17-hydroxypregenolone [42], estradiol, estriol [41,51–53], pregnenolone [42], progesterone [15,44,48], and testosterone [15,29,39,44,48,54,55].

Chromatographic separation plays an important role in the method performance [18]. 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 [56], and a complete specificity is therefore not possible. In addition to the chromatography, the sample preparation is also a critical aspect. Different techniques are used, such as liquid–liquid extraction [55] and solid phase extraction [57], normally with a protein precipitation step beforehand. For methodological details, the reader is referred to the corresponding publications.

Once more widely available, LC–MS/MS will become the preferred method for evaluation of gonadal steroids and adrenal steroids in children. The mass spectrometry-based methods for the steroids have been established as higher reference method procedures that will resolve the issues of high sensitivity measurements for steroids. As continued improvements are made with mass spectrometry technology including reduced sample requirements and improving test volume throughput, this approach to steroid testing should predominate in the years ahead. These methods can provide improved and optimal specificity and accuracy and minimize interferences from other steroid molecules. However, standardization of LC–MS/MS methods across laboratories needs to be established as it does for testosterone [58]. Appropriate standardization and reference materials are necessary to generate results that are interchangeable between laboratories and methods.

4.2. Steroid profiling

Above all the increased accuracy and specificity in comparison to immunoassay an essential strength of the HPLC–MS/MS technology for laboratory medicine, is the possibility for multiparametric analyses, the ability to quantitate a large number of analytes in a single scalable measurement process. All of the critical steroid metabolites can be analyzed simultaneously in a single assay. This is one of the seminal advantages of LC–MS/MS and is highly advantageous in the pediatric setting, as only small amounts of plasma enable determination of a whole profile of diagnostically important steroid hormones with highest specificity. Several studies [15,32,42,44,48,59,60] illustrate the usefulness of steroid profiles and provided further reason to develop methodologies for profiling steroids and metabolites with the goal of forming a common procedure able to distinguish almost all steroid-related disorders.

Congenital adreanal hyperplasia (CAH) is caused by a group of autosomal recessive disorders of adrenal cortisol biosynthesis. Defective mineralocorticoid synthesis may lead to life-threatening salt-wasting crisis [61,62]. In congenital adrenal hyperplasia, the defects occur in one of the enzymatic steps required to synthesize cortisol from cholesterol in the adrenal gland. Because of the impaired cortisol secretion, adrenocorticotropic hormone levels rise due to impairment of a negative feedback system, which results in hyperplasia of the adrenal cortex. Excess production of hormones proximal to the enzymatic defect results in various clinical phenotypes. In the Caucasian population, 21-hydroxylase deficiency (CYP21A2), the classical form of CAH, accounts for more than 90% of all cases, whereas 5% are caused by 11-hydroxylase deficiency (CYP11B1). Other enzyme deficiencies and clinical phenotypes are less frequent.

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 affected female fetus genitalia in the classical form of CAH. Lifelong hormone replacement is required.

Plasma steroid profiles allow for the acquisition of more clinically useful data than can be obtained through the measurement of a single steroid alone [32,44]. For example, the spectrum of diagnostically important metabolites in a plasma steroid profile is particularly helpful concerning the differential diagnosis of ambiguous genitalia in the newborn because it can delineate disorders associated with virilization (21-hydroxylase deficiency, 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency in females). In Fig. 1 examples for such steroid profiles are shown: a patient with 17-hydroxylase/17,20 lyase deficiency (A), a CAH patient with 21-hydroxylase deficiency (B). The steroid profile of the patient with 17-hydroxylase/17,20 lyase deficiency is dominated by corticosterone lacking cortisol and cortisone.

4.3. Newborn screening

The principal concerns for clinicians managing CAH are to make timely diagnoses of affected individuals, treat hormone deficiency appropriately, handle the problems accompanying genital ambiguity, and avoid long-term morbidities. Neonatal screening for CAH was first introduced in 1977 when immunoassays became available. Newborn screening allows the diagnosis of congenital adrenal hyperplasia (CAH) before symptoms appear preventing the severe and potentially life-threatening crisis associated with this disease in infancy. Diagnosis of CAH relies mainly on the measurement of plasma concentrations of 17-OH-progesterone. Most pediatric cases of 21-hydroxylase deficiency have grossly elevated concentrations. Modest increase in 17-OH-progesterone can also occur in deficiencies of 11β-hydroxylase and 3β-hydroxysteroiddehydrogenase. Traditional screening by enzyme immunoassay (EIA) results in a large number of false positives. Stressed normal newborn babies may have 17-OH-progesterone concentrations as high as 100 nmol/l. Also preterm babies often show high levels of 17-OH-progesterone because of stress or delayed maturation of 11-hydroxylase and this, together with interference in the assay by fetal adrenal zone steroid sulfates, can lead to diagnostic confusion if 17-OH-progesterone is measured in the first days after birth [63]. Compared with other neonatal screening tests, the specificity of screening for 21-CAH by immunoassay is low. In addition, increased serum concentrations of 17-OH-progesterone are associated with low birth weight and illness, causing an even higher incidence of false-positives in the premature and sick infant population. Adjustment the cut of 17-OH-progesterone for gestational age and birth weight improves the specificity of the test [64]. Despite this improvement, CAH screening has the lowest predictive value among newborn screening tests when measurement of 17-OH-progesterone concentration alone is used.

To reduce the number of unnecessary tests, anxiety to families and physicians, and the burden to the newborn screening follow-up program, several groups [65–67] implemented a second tier test for CAH using steroid profiling by liquid chromatography–tandem mass spectrometry. Using steroid profiles they could minimize false-positive results attributable to cross-reactivity.

For example, the group of Sander [66,68] applied a method which allows rapid quantitative analysis of the steroids 21-deoxycortisol, 17-hydroxy-progesterone, and cortisol as downstream product. A sensitive ratio can be used to distinguish between true 21-CAH and false-positive results. The LC–MS/MS method allows immediate confirmation of 21-hydroxylase deficiency, because only a small amount of material is needed, which can be taken from the original filter card. Using a similar approach Schwarz et al. [67] could reduce the false-positive rate from 2.6% to 0.09%.

4.4. Saliva assays

Salivary hormone analysis is a proven alternative to plasma [69,70]. The non-invasive collection is convenient for diagnostic in children or out-patient sampling [71,72]. 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 conveniently collect samples themselves, if required. Salivary steroid levels can reflect the circulating level of free steroid rather than total circulating levels, which are confounded by the presence of circulating high affinity binding proteins.

The main goal in congenital adrenal hyperplasia (CAH) therapy is to approach the physiological replacement of cortisol deficiency while suppressing adrenal androgen overproduction. Currently, standard medical treatment of CAH consists of giving a glucocorticoid (a cortisol-like steroid medication, e.g., oral hydrocortisone in children, or prednisone or dexamethasone in older patients). Untreated individuals will experience virilization, precocious pseudopuberty, and in salt wasting CAH form, salt losing crisis and dehydration. Patients who had bad metabolic control during infancy and adolescence will finally have reduced adult height. In addition female subjects may have menstrual disturbances and infertility.

The diurnal variation of plasma 17-OH-progesterone is reflected by similar changes in salivary 17-OH-progesterone and hence timed salivary 17-OH-progesterone has been used in diagnostic setting [28,73]. Early morning salivary 17-OH-progesterone is helpful to control the hormone replacement therapy and patient compliance.

However, a major disadvantage in the use of saliva is the low 17-OH-progesterone concentration, and only a few immunoassays, which were primarily developed for the serum 17-OH-progesterone, have been applied to the measurement of the salivary 17-OH-progesterone. Due to the rather low concentration of 17-OH-progesterone in saliva, determination of the steroid has not always been easy and requires sensitive and highly specific assay techniques. Due to variability in tracer preparation RIAs have the disadvantage of changing sensitivity and therefore questionable reliability during day-to-day clinical application. For these assays, care is required to address standardization issues as well as differing matrices of serum and saliva [74]. Using the same method for plasma and saliva is a great advantage of mass spectrometry. No matrix effects or cross-reactivity must be considered [18].

Tandem mass spectrometry is also used to analyse cortisol and cortisone in saliva [75]. Salivary cortisol determinations have proved popular in psychobiology, stress and sports medicine studies [76]. 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 [77].

Antenatal betamethasone administration in pregnancies at risk for preterm delivery before 34 weeks of gestation is an established and effective procedure to improve neonatal outcome by decreasing neonatal mortality and infant morbidity. However, in different animal models, excessive prenatal glucocorticoid administration reduces birth weight and increases blood pressure and alters the activity of the HPA axis in adults. Saliva is an excellent medium to analyze such changes in the hypothalamus-pituitary-adrenal axis. The results of Schaffer et al. [78] indicate that a single course of antenatal betamethasone treatment induces a suppression of

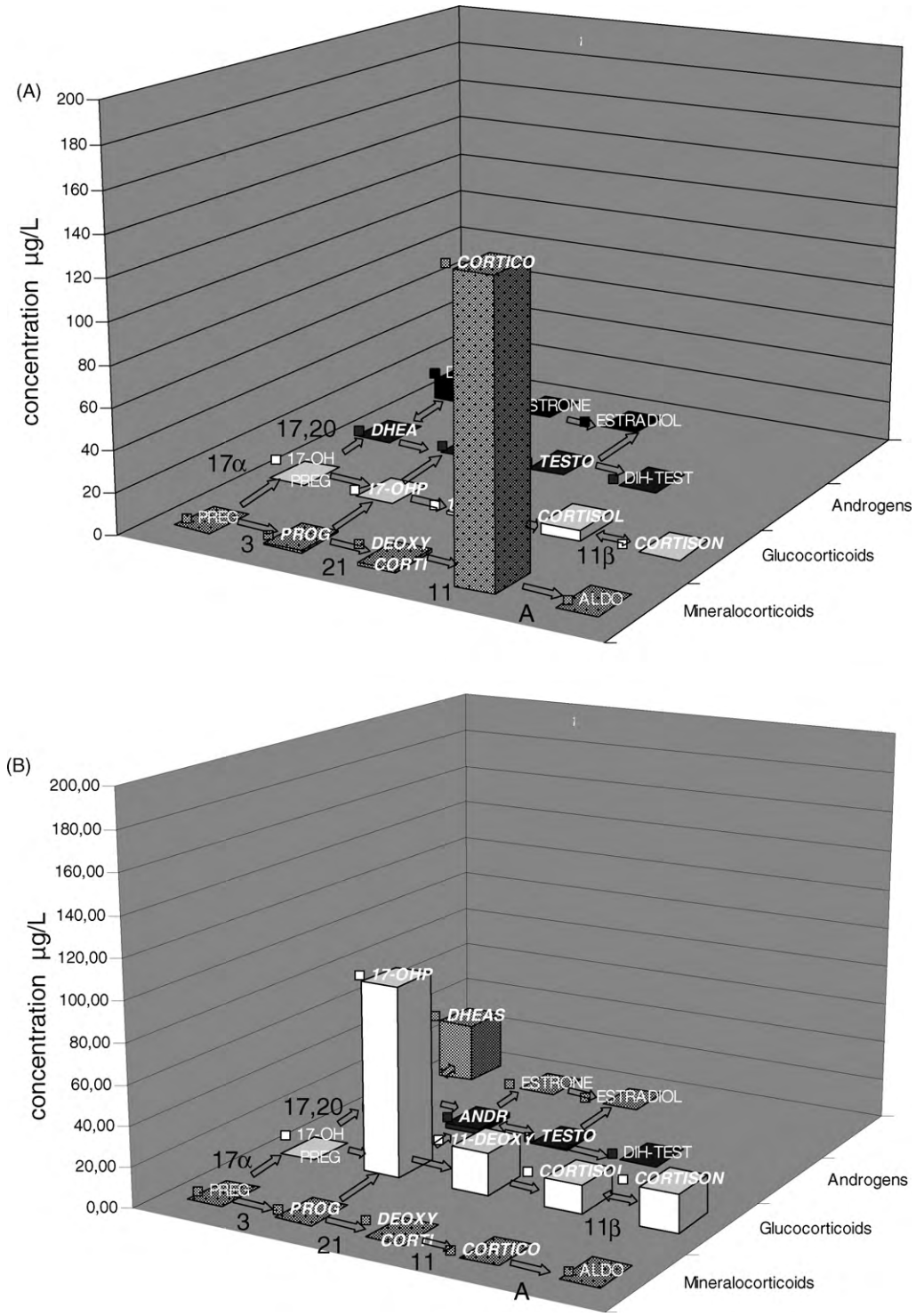

Fig. 1. Plasma steroid profiles of CAH patients: (A) patient with 17-hydroxylase deficiency (B) patient with 21-hydroxylase deficiency. Steroids: PREG pregnenolone (not measured); 17-OH-PREG: 17-hydroxypregnenolone (not measured); DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulphate; PROG: progesterone; 17-OHP: 17-hydroxyprogesterone; ANDR: androstenedione; ESTRONE, estrone (not measured); DEOXYCORTI: deoxycorticosterone; 11-DEOXY: 11-deoxycortisol; TESTO: testosterone; ESTRADIOL: estradiol (not measured); CORTICO: corticosterone; CORTISOL: cortisol; CORTISON: cortisone; DIH-TEST: dihydrotestosterone; ALDO: aldosterone (not measured). *DHEAS µg/dl enzymes: **3**, 3β-hydroxysteroid dehydrogenase, **21**, 21-hydroxylase, **11**, 11β-hydroxylase, **17α**, 17α-hydroxylase, **17,20**, 17,20-lyase, **11β**, 11β-hydroxysteroiddehydrogenase, **A,** two-step process of aldosterone synthesis.

stress reactivity in healthy newborns. Whether the observed effects transform into long-term consequences has to be established by follow-up studies.

Implementation of the new assays in diagnostic practice, depends on the availability of new reference intervals, as the new methods are often more specific compared to the older techniques.

4.5. Urine assays

Only a small amount of circulating plasma steroids is excreted unchanged in the urine, the remainder is metabolized by the liver. A large number of hepatic metabolites of each steroid is produced, most containing additional hydroxyl groups and linked to a sul-

phate or glucuronide moiety, rendering them more soluble and readily excretable by the kidney. A great deal is known about various urinary metabolites of the circulating steroids because their measurement in pooled 24-h urine samples has been an important means of studying adrenal steroids [79–82].

The potential use of LC–MS/MS for steroid conjugate analysis is promising. LC–MS/MS offers the possibility for measurement of intact steroid conjugates and related compounds. An example of the potential use has been demonstrated by Raffaelli and co-workers [83] for direct determination of the ratio of tetrahydrocortisol + allo-tetrahydrocortisol to tetrahydrocortison. The use of liquid chromatography/tandem mass spectrometry (LC/MS/MS) can give several benefits in the urinary detection of steroids such as avoiding the derivatization step or in particular the hydrolysis of steroid conjugates prior to analysis [84,85].

A lot of work is still necessary in order to improve both reliability and ruggedness. In fact, urine is particularly rich of different steroidic components, only a small part of which is in free form, being the major fraction constituted by glucuronide and sulphate conjugates. Most fragment ions observed in their tandem mass spectra are common to different components, therefore a complete specificity is not possible [86]. For these reasons the HPLC method must be able to separate in a satisfactory way the components of interest. Due to the complicated nature of the matrix and possible suppression effects, the use of deuterated internal standards is necessary to assure optimal accuracy. Nevertheless the described assays are a good starting point for setup sensitive rapid methods on routine bases.

5. Conclusions

Tandem mass spectrometry usually offers high specificity, even in complex sample matrices, it can simultaneously measure several steroids in serum and other matrices with minimal sample preparation. It can be routinely employed in a clinical environment and is attractive because of its simplification of sample processing and high throughput. Mass spectrometry can be regarded as a complementary technique having technical advantages over immunoassays for specific application. As continued improvements are made with mass spectrometry, this approach to steroid testing should predominate in the years ahead. The ability of these mass spectrometry methods to detect low levels of steroids in children enhances their clinical use particularly at extremely low levels of concentration. Efforts must be made to appropriately standardized and calibrated methods. The establishing of new reference intervals is a prerequisite for implementation of these methods in diagnostic practice. Steroid profiles open up new vistas.

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