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An improved micro-method for the measurement of steroid profiles by APPI-LC-MS/MS and its use in assessing diurnal effects on steroid concentrations and optimizing the diagnosis and treatment of adrenal insufficiency and CAH

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Abstract

Our goals were to 1) develop an improved micro-method usable for neonates for steroid profile measurements and a method to measure androsterone, a key steroid in the recently described androgen backdoor pathway together, with dehydroepiandrosterone and 2) to assess if dehydroepiandrosterone diurnal concentration fluctuations exist potentially necessitating strict adherence to time of blood sample draw and requirement of separate time-dependent reference intervals. Liquid chromatography-tandem mass spectrometry was performed with an atmospheric pressure photoionization source [1]. For each sample 50 μ L (100 μ L for the backdoor pathway) of serum was deproteinized by adding 75 μ L (150 μ L for the backdoor pathway) of acetonitrile containing the internal standards. After centrifugation, 75 μ L (150 μ L for the backdoor pathway) of supernatant was diluted with 250 μ L of water and injected onto a Poroshell 120 EC-C8 column (SB-C8 column for the backdoor pathway). Within-run coefficients of variation ranged from 2.4–10.4% and between-day coefficients of variation from 2.9–11.2%. Comparison studies yielded correlation coefficient between 0.97 and 1.00 with recoveries of 90% or greater. Our methods analyze a 9 steroid profile and an additional 2 steroid profile (backdoor pathway) with minimal sample volume (usable in neonates optimizing early diagnosis of endocrinopathies and genetic

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diseases). Low limits of quantitation make these methods ideal for steroid measurement in women and prepubertal children. As diurnal variations of dehydroepiandrosterone and other steroids [2] concentrations are clinically significant we recommend that separate reference intervals be developed for 8 am, 8 pm, and midnight sample draws. The use of this approach in improving the diagnosis of patients with adrenal insufficiency and congenital adrenal hyperplasia is discussed.

Keywords

Micro-method atmospheric pressure photoionization LC-MS/MS; Steroids; Diurnal variation; Dehydroepiandrosterone

1. Introduction

A number of tandem-mass spectrometry based methods for the measurement of steroids have been reported in the literature [3–8]. With accurate steroid quantitation becoming the gold standard, specific LC-MS/MS methodologies are replacing the older and less specific HPLC [9] and immunoassay approaches [10]. The latter have been frequently found to correlate poorly with mass spectrometry methods at low steroid concentrations [11].

We previously reported a nine-steroid profile [12] and a twelve-steroid profile [13] employing tandem mass spectrometry utilizing the ABSciex API-3000 and API-5000, respectively, with an atmospheric pressure photoionization source (APPI). Measurement of a steroid panel by LC-APPI-MS/MS permits investigation into causes of hyper- and hypo-androgenism in conditions such as congenital adrenal hyperplasia (CAH), polycystic ovary syndrome (PCOS), or adrenal disorders quickly and accurately. A profile looking at cortisol, 17 α -hydroxyprogesterone (17-OHP), and androstenedione has been recommended for diagnosis and assessment of treatment for CAH [9] while we have previously shown that adrenal insufficiency (AI) diseases such as Addison's disease are best assessed through measurement of aldosterone, cortisol, dehydroepiandrosterone (DHEA) and 11-deoxycortisol (11-DOC) after an adrenocorticotrophic hormone (ACTH) stimulation test [14]. Having a profile assay that provides measurement of a more comprehensive panel of steroids permits more accurate diagnosis and assessment of treatment for many endocrine/genetic disorders [11]. Classic salt wasting CAH most commonly presents in the neonatal period. Preterm and term neonates have very limited blood volume, with sick neonates even more likely to be anemic and require transfusions. Thus blood volume that can be sampled for diagnostic testing is very limited. Guidelines for neonates' phlebotomy recommend trial-related blood loss should not exceed 3% of the total blood volume during a period of four weeks and should not exceed 1% at any single time [15]. A micro method requiring 50 μ L of serum/plasma to perform a multi-steroid panel would allow for early diagnosis as well as prevent incidence of anemia related to blood draw in neonates. Using this 3rd generation method we have recently explored the role of this approach to optimize the treatment of patients with CAH [16]. Furthermore the use of the APPI source has been recommended because the signal to noise ratio is better than that obtained when using ESI. This is because APPI is a soft ionization source and essentially ionizes steroids and little else. As fewer ions are created there is also a diminished ionization inhibitory effect. For example, if you follow

the peak areas of the internal standards used in patient samples they vary by plus minus 15% of the mean value with APPI but can vary by a factor of 30 with ESI [1].

The major route of androgen synthesis in humans is the classic Δ^5 pathway. In this pathway, the 17,20 lyase activity of CYP17A1 leads to the conversion of 17-hydroxypregnenolone which gets converted into DHEA. DHEA is metabolized to androstenedione and then to testosterone [17]. Testosterone is converted into dihydrotestosterone (DHT), the more potent androgen [18]. Patients with 21-hydroxylase deficiency (21-OHD), the most common form of CAH, have a mutation in the CYP21A2 gene that causes a significant build up of 17-OHP [19]. This build up of 17-OHP can cause an increase in DHT production via the “backdoor” pathway. 17-OHP can be converted to 5 α -pregnane-3 α ,17 α -diol-20-one (pdiol) by the activities of 5 α -reductase and 3 α -hydroxysteroid dehydrogenase (HSD) [20]. Androsterone is synthesized by the action of the CYP17A1 enzyme due to its high affinity for pdiol, a substrate for its 17,20 lyase activity [20–23]. Pdiol is a better substrate for CYP17A1 than are either 17-OHP or 17-hydroxypregnenolone and is superior to testosterone as a substrate for 5 α -reductase making the alternative backdoor pathway a possibility for DHT production in patients with 21-OHD [21,22].

The objectives of our study were to develop 1) improved micro-methods for steroid profile measurements usable for neonates, 2) validate a method to measure androsterone, a key steroid in the recently described androgen backdoor pathway, together with DHEA, 3) demonstrate and define the extent of DHEA diurnal concentration fluctuations potentially necessitating strict adherence to time of blood sample draw and requirement of separate time-dependent reference intervals and 4) optimize diagnosis and treatment of patients with AI and CAH.

2. Materials and methods

2.1. Chemicals and reagents

Corticosterone, 11-deoxycortisol, androstenedione, testosterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, progesterone, cortisol, cortisone, 21-deoxycortisol, androsterone, hydrocortisone-9,11,12,12-d₄ (cortisol-d₄) and HPLC grade toluene were from Sigma-Aldrich. 4-Pregnen-11 β ,21-diol-3,20-dione-2,2,4,6,6,17 α ,21,21-d₈ (corticosterone-d₈), 4-Pregnen-17 α ,21-diol-3,20-dione-21,21-d₂ (11-deoxycortisol-d₂), 4-Androsten-3,17-dione-2,2,4,6,6,16,16-d₇ (androstenedione-d₇), testosterone-1,2-d₂ (testosterone-d₂), 4-Pregnen-17 α -ol-3,20-dione-2,2,4,6,6,21,21,21-d₈ (17 α -hydroxyprogesterone-d₈) and progesterone-2,2,4,6,6,17 α ,21,21,21-d₉ (progesterone-d₉) were from C/D/N Isotopes. HPLC-grade methanol and HPLC-grade acetonitrile were from Fisher Scientific. Deionized water was prepared from Millipore Synergy UV water system. BioCon II CD charcoal stripped human plasma was from Bioresource Technology.

2.2. Preparation of stock solutions, internal standards, and controls

Stock solutions of each analyte and internal standards (IS) were prepared separately at a concentration of 1.0 mg/mL in methanol. Sub-stocks were prepared by mixing appropriate volumes of individual analyte stock solutions to obtain concentrations of 10 μ g/mL for

corticosterone, testosterone, 17 α -hydroxyprogesterone, DHEA, 21-deoxycortisol and progesterone, 5.0 μ g/mL for 11-deoxycortisol, androsterone and androstenedione, and 1.0 μ g/mL for cortisol and cortisone. Working stocks were prepared in methanol by mixing the appropriate volumes of individual analyte sub-stock solutions to obtain concentrations of 0.5 μ g/mL for 11-deoxycortisol and androstenedione, 1.0 μ g/mL for corticosterone, testosterone, 17 α -hydroxyprogesterone, 21-deoxycortisol, androsterone, and DHEA, 2.0 μ g/mL for progesterone, 10.0 μ g/mL for cortisone, and 50.0 μ g/mL for cortisol. A six point calibration curve (plus blank) was prepared by mixing the appropriate volumes of individual working stock solutions with BioCon II Human Serum, to a total volume of 40 mL, to obtain calibrator S6, with the concentrations of 4.0 ng/mL for androsterone, 5.0 ng/mL for 11-deoxycortisol and androstenedione, 10.0 ng/mL for testosterone, 17 α -hydroxyprogesterone and DHEA, 12.0 ng/mL for corticosterone, 20.0 ng/mL for progesterone and 21-deoxycortisol, 100.0 ng/mL for cortisone, and 600.0 ng/mL for cortisol. The other five calibrators were prepared by diluting the S6 solution with BioCon II Human Serum.

Stock IS solutions of 1.0 mg/mL were diluted with acetonitrile to produce the IS solution containing 2.0 ng/mL androstenedione-d₇, 2.5 ng/mL 11-deoxycortisol-d₂ and testosterone-d₂, 3.0 ng/mL 17 α -hydroxyprogesterone-d₈, 4.5 ng/mL corticosterone-d₈, 5.0 ng/mL progesterone-d₉, and 150.0 ng/mL cortisol-d₄. Quality control materials at three levels were purchased from Bio-Rad Laboratories.

2.3. Specimens

Serum samples, for method comparison studies, with results for each steroid were kindly provided by the Mayo Clinic, Rochester, MN; Children's National Medical Center, Washington, DC; and NMS Labs, Willow Grove, PA. The NIH samples reported in this study were approved by the institutional review board of NIH, approved protocol 93-CC-0094, or by the NIDDK/NIAMS institutional review board, approved protocol NCT00428987.

2.4. Sample preparation

For the 9 steroid panel method, 50 μ L of serum was deproteinized by adding 75 μ L of acetonitrile containing internal standards. After centrifugation, 75 μ L of supernatant was diluted with 250 μ L of water and a 100 μ L aliquot was injected onto a Poroshell 120 EC-C8 column. For the backdoor pathway, 2 steroid method, 100 μ L of serum were deproteinized by adding 150 μ L of acetonitrile containing internal standards. After centrifugation, 150 μ L of supernatant was diluted with 250 μ L of water and a 300 μ L aliquot was injected onto a Poroshell 120 SB-C8 column. Sample preparation was performed at room temperature.

2.5. LC-APPI-MS/MS

An Agilent 6490 Triple Quadrupole mass spectrometer with iFunnel technology (Agilent Technologies) operated in positive mode under MRM conditions equipped with an APPI source, a 1260 infinity high performance autosampler, a 1290 infinity thermostat column compartment (TCC), and a 1290 infinity binary pump were used to perform the analysis. The MRM transitions used to identify and quantify each analyte and its labeled internal standard are listed in table 1. The APPI photomate system uses a krypton lamp that emits

photons at 10.0 and 10.6 eV and an 1100 series isocratic pump and degasser for its dopant (toluene) delivery. Nitrogen gas from a nitrogen generator (Peak Scientific, model Genius 3010) was heated and used in the APPI source to desolve and concentrate ions for transfer into the mass spectrometer. Ultra high purity compressed nitrogen (Roberts oxygen company) was used in the curved collision cell, which helps to reduce noise, to produce product ions. Unit mass resolution was used in both MS1 and MS2. Data were acquired and processed by Mass Hunter workstation software (Agilent Technologies). Chromatographic separation was carried out using a reversed phase Agilent Poroshell 120 EC-C8 analytical column (2.1×30 mm, $2.7 \mu\text{m}$ particle size) for the 9 steroid panel, and a Poroshell 120 SB-C8 analytical column (4.6×50 mm, $2.7 \mu\text{m}$ particle size) for the “backdoor pathway” method, held at 40°C with a 1290 TCC.

Our LC-MS/MS procedure is based on an online cleaning and extraction of the injected deproteinized diluted serum sample with subsequent introduction into the mass spectrometer through an internal switching valve. Once the processed sample was injected onto the column it was held and washed with buffer A (methanol: water, 2:98, v/v) for 3 minutes. After column washing the steroids were eluted using a methanol gradient as follows: For the 9 steroid panel: 50% B (methanol: water, 98:2, v/v) to 58% B over 3 minutes, 58% B to 90% B over 1 minute, holding at 90% B for 1.5 minutes, and finally 90% B to 20% B in 0.01 minutes and holding for 3 minutes, with a constant flow rate of 0.65 mL/min. For the backdoor pathway 2 steroid method: 57% B to 90% B over 4.5 minutes, holding at 90% B for 2 minutes, and finally 90% B to 20% B in 0.01 minutes and holding for 3 minutes, with a constant flow rate of 0.70 mL/min. For the 9 steroid panel the toluene was delivered into the APPI photomate source at a continuous flow rate of 0.12 mL/min (0.09 mL/min for the Backdoor pathway). The compound-dependent parameters of individual steroid analytes are listed in table 1. Refer to figure 1 for each steroid MRM chromatograms.

Calibrations, using internal standards, were done by linear regression analysis. Correlation coefficient (r-values) for each of the steroids ranged between 0.990 and 0.999. The peak area ratio between target analyte and its respective internal standard was used for quantitation.

2.6. Accuracy/comparability and imprecision

The accuracy of the method was evaluated by two methods: (a) comparison studies to LC-MS/MS methods applied at Mayo Clinic, NMS Labs, and Children’s National Medical Center and (b) performing recovery studies (Table 2). The imprecision was evaluated by assaying Bio-Liquichek Plus Control replicates at 2 or 3 different concentration levels ($n = 20$ for within-day and between-day) (Table 3). Linear regression and Deming regression analysis were performed.

2.7. Effect of time of day on steroid concentrations measured

We examined the effects of diurnal variation for DHEA on healthy individuals. Midnight samples ($n = 19$) were collected between 11:30 pm to 12:00 am and morning samples ($n = 19$) were collected the following morning between 7:30 am and 8:00 am. Healthy controls were not on any medications or supplements, had stable body weight (within 3%) for at least 30 days prior to testing, and were generally healthy with no indication of current or past

disease during a history and physical examination performed by a medical provider. Additionally, all females were pre-menopausal and in their early follicular phase during their inpatient admission based on their menstrual cycle histories. Paired sample Student's T-test was performed between am and pm values.

2.8 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software) and MedCalc version 15.11.3 (MedCalc Software). Significant values were determined at $p < 0.05$.

3. Results and Discussion

Figure 1 shows the chromatograms obtained for each of the steroids in a standard solution using Agilent's Poroshell columns. The gradients were optimized to yield the maximum signal response and peak resolution for each analyte. Each analyte and internal standard's specific transition and collision energies (Table 1) were obtained by using 1.0 µg/mL solutions, individually injected into the tandem mass spectrometer, and the Mass Hunter optimizer software. All MRM transitions were obtained in the positive mode. The main working parameters for the mass spectrometer are: gas temperature of 325°C for the steroid panel (225°C for the backdoor pathway); vaporizer temperature of 400°C for the steroid panel (425°C for the backdoor pathway); gas flow of 11 L/min for both methods; nebulizer pressure of 60 psi for both methods; capillary voltage of 4000 for the steroid panel (2000 for the backdoor pathway); and dwell time of 20 ms for the steroid panel (70 ms for the backdoor pathway). The first, second, and third generation steroid profile methods have chromatographic times of 18.0, 11.0, and 7.0 minutes respectively. The corresponding sample volumes were 760, 200, and 50 µL respectively. The DHEA/androsterone backdoor pathway method described allow for simultaneous quantitation in positive-ion mode by tandem mass spectrometry within 8.5 minutes. The enhanced sensitivity provided by the Agilent 6490 tandem mass spectrometer allowed us to reduce the injection volume of 1000 µL in our 2nd generation method to 100 µL in the steroid method and 300 µL in the backdoor pathway method. Further reduction of sample injection volume utilizing the APPI source on the Agilent 6490 is unlikely to provide sufficient sensitivity for several of the steroids. However, moving the assay onto the ABSciex 6500 will provide the necessary signal increase to reduce the injection volume another ten-fold. We replaced the supelco C-8 column with Agilent's poroshell series columns, giving better peak resolution [14].

Correlation values comparing our method with LC-MS/MS based methods performed at the Mayo Clinic (M), Children's National Medical Center (CNMC), and National Medical Services Labs (NMS) for the steroids are; Testosterone [Sy.x = 29.4, NIH=0.957*CNMC +0.518, r=0.990], DHEA [Sy.x = 0.41, NIH=1.094*M-0.443, r=0.998], cortisol [Sy.x = 1.2, NIH=1.032*NMS+0.001, r=0.968], progesterone [Sy.x = 1.3, NIH=1.007*CNMC+0.161, r=0.984], 17α-hydroxyprogesterone [Sy.x = 24.0, NIH=1.012*CNMC+6.179, r=0.998], 11-deoxycortisol [Sy.x = 6.2, NIH=0.895*CNMC+3.759, r=0.995], androstenedione [Sy.x = 9.4, NIH=1.028*CNMC+1.113, r=0.985], corticosterone [Sy.x = 49.7, NIH=1.112*CNMC-24.500, r=0.991], cortisone [Sy.x = 1.0, NIH=0.970*M-0.381, r=1.000].

The r-values obtained were excellent, ranging between 0.968 and 1.000. The within-run and between-day imprecision at three concentration levels are shown in Table 3. The between-day coefficients of variation (CVs) ranged from 3.7% to 11.1% at the low concentration level and 2.9% to 8.2% at the high concentration level. The within-run CVs ranged from 2.8% to 10.2% at the low concentration level and 2.4% to 10.4% at the high concentration level (Table 3). The accuracy was evaluated using two approaches; first, the addition of known amounts of each steroid were added to a charcoal treated pool of plasma (Table 2) and, secondly, by method comparison. The mean recovery of each steroid was determined at a low and high concentration level with replicates of $n = 5$. Table 2 shows recoveries ranging from 90% to 105% at the low level and 98% to 101% at the high level. The lower limit of detection (LOD = defined as a signal to noise ratio of 3) for each steroid was: cortisone 0.60 ng/mL, cortisol 0.60 ng/mL, corticosterone 0.08 ng/mL, 11-deoxycortisol 0.02 ng/mL, androstenedione 0.08 ng/mL, testosterone 0.05 ng/mL, 17 α -hydroxyprogesterone 0.08 ng/mL, progesterone 0.01 ng/mL, DHEA 0.30 ng/mL, androsterone 0.13 ng/mL and 21-deoxycortisol 0.09 ng/mL. The lower limit of quantitation (LOQ = defined as a signal to noise ratio of 5) for each steroid was: cortisone 1.00 ng/mL, cortisol 1.00 ng/mL, corticosterone 0.13 ng/mL, 11-deoxycortisol 0.04 ng/mL, androstenedione 0.13 ng/mL, testosterone 0.08 ng/mL, 17 α -hydroxyprogesterone 0.13 ng/mL, progesterone 0.01 ng/mL, DHEA 0.50 ng/mL, androsterone 0.22 ng/mL and 21-deoxycortisol 0.14 ng/mL. Excellent specificity was obtained through use of MRM's, optimization of columns, and chromatographic conditions. The method utilizes a slow gradient, increasing the organic phase slowly from 50% to 58% over a period of 3 minutes to ensure maximum separation of compounds with the same mass transition. To minimize the chance of interferences the method allows for sufficient degree of chromatographic separation at the price of a prolonged analysis time. Ion suppression was evaluated by comparing internal standard areas/peak heights between patient samples and calibrators. The method has been used routinely in our clinical laboratory for over 18 months (>8,000 patient samples).

The literature has clearly documented variation in testosterone and cortisol concentrations with time of day [2,24–31] but less information was available for other steroids. The effect of the different collection times on DHEA concentrations can be seen in Figure 2. DHEA reference intervals for 20–60 year old adult males and females are identical (25–625 ng/dL). There were unfortunately only 19 controls with these timed samples available to us, of which 10 were female and 9 male. Means of the males and females were almost identical and so these groups were combined. Our findings revealed significant diurnal fluctuations for DHEA ($p < 0.0001$) showing significantly higher values in the morning versus the midnight draws (Fig. 2). DHEA mean value increased from 230.7 ng/dL at midnight to 566.4 ng/dL at 08:00 AM.

AI and CAH have both been studied using our approach. AI is best diagnosed by measurement of aldosterone, DHEA and 11-DOC after performing an ACTH stimulation test. These three analytes are all superior to the measurement of cortisol [14]. In CAH measurement of 17-OHP, cortisol, androstenedione and DHEA have allowed for optimization of the patients dose regimen leading to improved patient care [16].

4. Conclusion

We have developed a 3rd generation steroid profile assay utilizing an LC-APPI-MS/MS based method for the analysis of 9 steroids with a 7.0 minute elution time and a 2 steroid “backdoor” pathway method with an 8.5 minute maximum elution time. These methods require as little as 50 µL of serum volume and a 100 µL injection volume. The methods correlate very well with LC-MS/MS based methods used at the Mayo Clinic, Children’s National Medical Center, and NMS Labs. By using an organic solvent protein precipitation coupled with LC-MS/MS methodology employing positive MRM mode the diagnosis of important endocrinopathies is faster thus allowing early diagnosis and treatment of diseases. Steroid profile monitoring is particularly useful as many endocrine disorders affect levels of several steroids and not just one. The inclusion of androsterone in our DHEA method allows us to assess the clinical use of the “backdoor” pathway for androgen synthesis and it’s role in various clinical situations. Use of a small sample volume permits assessment of underlying steroid abnormalities in neonates thereby optimizing early diagnosis of endocrine/genetic metabolic diseases. The protein crash and shoot approach is simple but doesn’t sacrifice high specificity and sensitivity and makes these methods attractive for widespread application in clinical laboratories. The significant diurnal fluctuation found in DHEA concentration demonstrates the need for standardization of blood collection times and follows our paper indicating significant diurnal variations for other steroids [2]. We also recommend time specific, as well as age and sex related, reference intervals be considered at times of 8 am, 8 pm, and midnight. Reference interval studies reported recently [24] and critiqued [32] in clinical chemistry have failed to adequately control for the diurnal fluctuations found in this study.

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Abbreviations

DHEA	Dehydroepiandrosterone
DHT	dihydrotestosterone
17 OHP	17α-hydroxyprogesterone
21-OHD	21-hydroxylase deficiency
CAH	congenital adrenal hyperplasia
HSD	3α-hydroxysteroid dehydrogenase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
PCOS	polycystic ovarian syndrome
IS	internal standard
APPI	atmospheric pressure photoionization

TCC	thermostat column compartment
LOQ	lower limit of quantitation
LOD	lower limit of detection
21-DOC	21-deoxycortisol
11-DOC	11-deoxycortisol
Pdiol	5 α -pregnane-3 α ,17 α -diol-20-one
ACTH	adrenocorticotrophic hormone
AI	adrenal insufficiency

References

1. Alary, JF. Comparative study: LC/MS/MS analysis of four steroid compounds using a new photo-ionization source and a conventional APCI source. Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics; 2001 May 27–31; Chicago. Santa Fe (NM): American Society for Mass Spectrometry; 2001. Abstract A010942
2. Stolze BR, Gounden V, Gu J, Abel BS, Merke DP, Skarulis MC, Soldin SJ. Use of micro-HPLC-MS/MS method to assess diurnal effects on steroid hormones [Letter]. Clin Chem. 2015; 61:556–8. [PubMed: 25518857]
3. Joos PE, Ryckeghem M. Liquid chromatography-tandem mass spectrometry of some anabolic steroids. Anal Chem. 1999; 71:4701–10. [PubMed: 10546536]
4. Dorgan JF, Fears TR, McMahon RP, Aronson FL, Patterson PH, Greenhut SF. Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. Steroids. 2002; 67:151–158. [PubMed: 11856538]
5. Chang YC, Li CM, Li LA, Jong SB, Liao PC, Chang LW. Quantitative measurement of male steroid hormones using automated on-line solid phase extraction-liquid chromatography-tandem mass spectrometry and comparison with radioimmunoassay. Analyst. 2003; 128:363–8. [PubMed: 12741642]
6. Tang PW, Law WC, Wan TS. Analysis of corticosteroids in equine urine by liquid chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl. 2001; 754:229–44. [PubMed: 11318419]
7. Taylor RL, Machacek D, Singh RJ. Validation of a high-throughput liquid chromatography-tandem mass spectrometry method for urinary cortisol and cortisone. Clin Chem. 2002; 48:1511–9. [PubMed: 12194928]
8. Minutti CZ, Lacey JM, Magera MJ, Hahn SH, McCann M, Schulze A, et al. Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab. 2004; 89:3687–93. [PubMed: 15292289]
9. Kautsky, MP. Steroid analysis by HPLC. New York: Marcel Dekker; 1981.
10. Abraham, GE. Radioassay systems in clinical endocrinology. New York: Marcel Dekker; 1981.
11. Soldin SJ, Soldin OP. Steroid hormone analysis by tandem mass spectrometry. Clin Chem. 2009; 55:1061–1066. [PubMed: 19325015]
12. Guo T, Chan M, Soldin SJ. Steroid profiles using liquid chromatography-tandem mass spectrometry with atmospheric pressure photoionization source. Arch Pathol Lab Med. 2004; 128:469–75. [PubMed: 15043485]
13. Guo T, Taylor R, Singh R, Soldin SJ. Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin Chim Acta. 2006; 372:76–82. [PubMed: 16707118]
14. Holst JP, Soldin SJ, Tractenberg RE, Guo T, Kundra P, Verbalis JG, et al. Use of steroid profiles in determining the cause of adrenal insufficiency. Steroids. 2007; 72:71–84. [PubMed: 17157339]

15. Howie SRC. Blood sample volumes in child health research: review of safe limits. *Bull World Health Organ.* 2011; 89:46–53. [PubMed: 21346890]
16. Debono M, Mallappa A, Gounden V, Nella AA, Harrison RF, Crutchfield CA, et al. Hormonal circadian rhythms in patients with congenital adrenal hyperplasia: identifying optimal monitoring times and novel disease biomarkers. *Eur J Endocrinol.* 2015 Dec; 173(6):727–37. [PubMed: 26340969]
17. Flück CE, Miller WL, Auchus RJ. The 17, 20-lyase activity of cytochrome P450c17 from human fetal testis favors the Δ^5 steroidogenic pathway. *Clin Endocrinol Metab.* 2003; 88:3762–3766.
18. Wilson JD. The role of 5 α -reduction in steroid hormone physiology. *Reprod Fertil Dev.* 2002; 13:673–678. [PubMed: 11999320]
19. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev.* 2000; 21:245–291. [PubMed: 10857554]
20. Wilson JD, Auchus RJ, Leihy MW, Guryev OL, Estabrook RW, Osborn SM, et al. 5 α -Androstane-3 α ,17 β -diol is formed in tammar wallaby pouch young testes by a pathway involving 5 α -pregnane-3 α ,17 α -diol-20-one as a key intermediate. *Endocrinology.* 2003; 144:575–580. [PubMed: 12538619]
21. Auchus RJ. The backdoor pathway to dihydrotestosterone. *Trends Endocrinol Metab.* 2004; 15:432–438. [PubMed: 15519890]
22. Gupta MK, Guryev OL, Auchus RJ. 5 α -Reduced C21 steroids are substrates for human cytochrome P450c17. *Arch Biochem Biophys.* 2003; 418:151–160. [PubMed: 14522586]
23. Mahendroo M, Wilson JD, Richardson JA, Auchus RJ. Steroid 5 α -reductase 1 promotes 5 α -androstane-3 α ,17 β -diol synthesis in immature mouse testes by two pathways. *Mol Cell Endocrinol.* 2004; 222:113–120. [PubMed: 15249131]
24. Kyriakopoulou L, Yazdanpanah M, Colantonio DA, Chan MK, Daly CH, Adeli K. A sensitive and rapid mass spectrometric method for the simultaneous measurement of eight steroid hormones and CALIPER pediatric reference intervals. *Clin Biochem.* 2013; 46:642–51. [PubMed: 23337690]
25. Brambilla DJ, Matsumoto AM, Araujo AB, McKinlay JB. The effect of diurnal variation on clinical measurement of serum testosterone and other sex hormone levels in men. *J Clin Endocrinol Metab.* 2009; 94:907–913. [PubMed: 19088162]
26. Cooke RR, McIntosh JE, McIntosh RP. Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model. *Clin Endocrinol.* 1993; 39:163–171.
27. Diver MJ, Imtiaz KE, Ahmad AM, Vora JP, Fraser WD. Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clin Endocrinol.* 2003; 58:710–717.
28. Faiman C, Winter JSD. Diurnal cycles in plasma FSH, testosterone and cortisol in men. *J Clin Endocrinol Metab.* 1971; 33:186–192. [PubMed: 4935634]
29. Nicolau GY, Haus E, Lakatua DJ, Bogdan C, Sackett-Lundeen L, Popescu E, et al. Circadian and circannual variations of FSH, LH, testosterone, dehydroepiandrosterone-sulfate (DHEA-S0 and 17-hydroxy progesterone (17 OH-Prog) in elderly men and women. *Endocrinologie.* 1985; 23:223–246. [PubMed: 2935925]
30. Winters SJ. Diurnal rhythm of testosterone and luteinizing hormone in hypogonadal. *J Androl.* 1991; 12:185–190. [PubMed: 1917682]
31. Rose RM, Gordon TP, Bernstein IS. Diurnal variation in plasma testosterone and cortisol in rhesus monkeys living in social groups. *J Endocrinol.* 1978; 76:67–74. [PubMed: 415103]
32. Gouden V, Soldin SJ. Clinical use of reference intervals derived from some CALIPER studies questioned. *Clin Chem.* 2014; 60:416–417. [PubMed: 24363369]

Highlights

- A micro-method for the measurement of a nine steroid profile.
- Micro-method for DHEA and androsterone, a steroid in the androgen backdoor pathway.
- Significant diurnal fluctuation in DHEA concentration from 8am to midnight.
- Time specific reference intervals and universal collection times recommended.

Figure 1a

Figure 1b**Figure 1.**

Chromatograms showing a serum sample, left side, and calibrator, right side, for each of the steroids in the methods described.

Figure 2. Box-and-whisker plots of midnight versus 8 am values for male and female samples for DHEA. Mean is indicated by small square markers and median by bar in box, $p < 0.0001$ ($p < 0.05$ is considered statistically significant).

Table 1

Tandem Mass Spectrometry: MRM conditions for all steroids.

Steroid	MRM Transition	Collision Energy (V)	Retention Time (min)
Cortisol	363.2/121.1	26	5.19
Cortisol-d4	367.3/121.0	26	5.19
Cortisone	361.2/163.0	22	5.13
11-deoxycortisol	347.3/97.1	30	5.48
11-deoxycortisol-d2	349.2/97.0	26	5.48
Corticosterone	347.2/121.2	22	5.44
Corticosterone-d8	355.3/337.2	14	5.44
17 α -hydroxyprogesterone	331.2/109.1	30	6.03
17 α -hydroxyprogesterone-d8	339.2/100.0	22	6.03
Progesterone	315.3/109.1	26	6.81
Progesterone-d9	324.3/100.0	22	6.81
Testosterone	289.1/109.0	22	5.86
Testosterone-d2	291.2/111.1	26	5.86
Androstenedione	287.1/97.1	18	5.64
Androstenedione-d7	294.2/100.1	26	5.64
Dehydroepiandrosterone	271.3/213.2	8	6.85
Androsterone	273.3/255.2	9	8.21
21-deoxycortisol	347.4/311.3	13	5.35

Table 2

Recovery from addition at two concentration levels (ng/mL) (n = 5)

Steroid	Blank	Conc. spiked	Mean conc. found	Difference (mean conc. – blank)	Recovery (%)
Cortisone	0.10	10.00	10.30	10.20	102.0
		100.00	100.00	100.00	100.0
Cortisol	0.61	72.00	72.40	17.80	99.7
		720.00	713.00	712.00	98.9
Corticosterone	0.05	0.48	0.49	0.44	92.0
		9.60	9.56	9.51	99.1
11-deoxycortisol	0.00	0.25	0.27	0.27	105.0
		5.00	5.05	5.05	101.0
Androstenedione	0.08	0.70	0.71	0.63	89.7
		7.00	7.10	7.02	100.0
Testosterone	0.01	0.45	0.45	0.44	98.2
		9.00	9.02	9.01	100.0
17 α -hydroxyprogesterone	0.03	0.45	0.44	0.41	90.1
		9.00	8.86	8.83	98.1
Progesterone	0.03	0.96	0.99	0.96	101
		19.20	19.20	19.10	99.7
DHEA	0.08	1.00	0.9	0.90	90.2
		12.00	12.10	12.00	100.0
Androsterone	0.03	1.00	0.97	0.94	94.2
		4.00	3.96	3.93	98.4
21-deoxycortisol	0.05	5.00	5.05	5.00	100.0
		10.00	9.75	9.70	97.0

Table 3
Within-run and between-day imprecision for each steroid using quality control samples (n = 20).

Steroid	Level 1		Level 2		Level 3	
	Mean, ng/mL	%CV	Mean, ng/mL	%CV	Mean, ng/mL	%CV
Corticosterone						
Within-run	0.65	10.2	1.27	8.10	NA	NA
Between-day	0.79	11.0	2.26	5.60	NA	NA
Cortisol						
Within-run	45.40	3.2	252.00	3.1	429.00	3.1
Between-day	43.50	7.7	221.00	5.0	400.00	5.5
Cortisone						
Within-run	6.06	4.8	9.23	6.6	NA	NA
Between-day	5.65	11.1	7.22	5.9	NA	NA
Testosterone						
Within-run	1.24	6.1	5.19	2.9	11.80	2.4
Between-day	1.21	9.1	4.71	6.9	10.50	6.4
Progesterone						
Within-run	0.65	9.3	8.12	2.5	16.00	3.2
Between-day	0.92	10.9	9.43	4.1	18.20	6.4
17 α -hydroxyprogesterone						
Within-run	0.63	3.8	2.35	7.4	4.70	6.2
Between-day	0.74	10.3	2.37	11.2	4.80	8.2
11-deoxycortisol						
Within-run	0.13	8.1	0.34	10.4	NA	NA
Between-day	0.13	10.7	0.33	7.7	NA	NA
Androstenedione						
Within-run	0.33	9.8	1.42	7.4	3.50	5.0

Steroid	Level 1		Level 2		Level 3	
	Mean, ng/mL	%CV	Mean, ng/mL	%CV	Mean, ng/mL	%CV
Between-day	0.37	8.6	1.42	9.9	3.70	7.9
DHEA						
Within-run	2.34	8.4	14.40	5.3	NA	NA
Between-day	2.39	9.9	13.50	7.6	NA	NA
Androsterone						
Within-run	0.51	2.8	2.00	3.3	NA	NA
Between-day	0.50	3.7	2.00	2.9	NA	NA
21-deoxycortisol						
Within-run	2.60	5.9	19.90	3.2	NA	NA
Between-day	2.60	6.0	20.10	3.0	NA	NA

^aNA; not available due to similar concentration of another level.