Received: 15 October 2021 | Revised: 13 December 2021 | Accepted: 19 December 2021

DOI: 10.1111/cen.14690

ORIGINAL ARTICLE

WILEY

# Diurnal salivary androstenedione and 17-hydroxyprogesterone levels in healthy volunteers for monitoring treatment efficacy of patients with congenital adrenal hyperplasia

Bas P. H. Adriaansen[1,2] | Johannes S. Kamphuis[3] | Mariska A. M. Schröder[2,4] | André J. Olthaar[1] | Carina Bock[1] | André Brandt[1] | Nike M. M. L. Stikkelbroeck[5] | Eef G. W. M. Lentjes[6] | Paul N. Span[7] | Fred C. G. J. Sweep[1] | Hedi L. Claahsen-van der Grinten[2] | Antonius E. van Herwaarden[1]

[1]Department of Laboratory Medicine, Radboud Institute of Health Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands

[2]Department of Paediatric Endocrinology, Amalia Children's Hospital, Radboud University Medical Centre, Nijmegen, The Netherlands

[3]Department of Clinical Chemistry and Haematology, Gelre Hospitals, Apeldoorn, The Netherlands

[4]Department of Laboratory Medicine, Radboud Institute of Molecular Life Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands

[5]Department of Endocrinology, Radboud University Medical Centre, Nijmegen, The Netherlands

[6]Central Diagnostic Laboratory, University Medical Centre Utrecht, Utrecht, The Netherlands

[7]Department of Radiation Oncology, Radboud University Medical Centre, Nijmegen, The Netherlands

**Correspondence**
Bas P. H. Adriaansen, Department of Laboratory Medicine, Radboud Institute of Health Sciences, Radboud University Medical Centre, 6525 Nijmegen, The Netherlands.
Email: bas.adriaansen@radboudumc.nl

## Abstract

**Objective:** Treatment of congenital adrenal hyperplasia (CAH) patients with glucocorticoids is often challenging since there is a delicate balance between over- and undertreatment. Treatment can be monitored noninvasively by measuring salivary androstenedione (A4) and 17-hydroxyprogesterone (17-OHP). Optimal treatment monitoring requires the establishment of reference values in saliva.

**Design:** A descriptive study.

**Patients:** For this study saliva of 255 healthy paediatric and adult volunteers with an age range of 4–75 years old was used.

**Measurements:** We developed a sensitive liquid chromatography-tandem mass spectrometry method, assessed salivary A4 and 17-OHP stability, and measured A4 and 17-OHP concentrations in saliva collected in the morning, afternoon, and evening.

**Results:** We quantified A4 and 17-OHP concentrations in the morning, afternoon, and evening and demonstrated that there is a significant rhythm with the highest levels in the morning and decreasing levels over the day. A4 and 17-OHP concentrations display an age-dependent pattern. These steroids remain stable in saliva at ambient temperature for up to 5 days.

**Conclusions:** Good stability of the steroids in saliva enables saliva collection by the patient at home. Since salivary A4 and 17-OHP display a diurnal rhythm and age-dependent pattern, we established reference values for both children and adults at three time points during the day. These reference values support treatment monitoring of children and adults with CAH.

KEYWORDS

17-hydroxyprogesterone, androstenedione, congenital adrenal hyperplasia, medication therapy management, reference values, saliva, tandem mass spectrometry

## 1 | INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by an enzymatic defect in adrenal steroidogenesis. In 95% of CAH, the deficient enzyme is 21-hydroxylase, leading to impaired production of cortisol, which results in decreased negative feedback on the pituitary gland and increased production of adrenocorticotropic hormone (ACTH). Consequently, the adrenal cortex is chronically stimulated, leading to increased production of cortisol precursors before the enzymatic block such as 17-hydroxyprogesterone (17-OHP), which is also partially shunted into the adrenal androgen synthesis pathway (e.g., androstenedione [A4]). Therefore, in addition to the potential risks of cortisol deficiency, this enzymatic block also leads to an excess of adrenal androgens with symptoms such as rapid postnatal growth with advanced bone age and premature pubarche in affected children. In girls, elevated androgens might lead to virilization of the external genitalia, menstrual disorders, and/or hirsutism.[1] In addition, elevated levels of ACTH may lead to the formation of testicular adrenal rest tumours.[2] Patients with mutations leading to a (near) complete loss of enzymatic function, usually associated with <1% residual activity, also have decreased concentrations of aldosterone, leading to a neonatal salt loss that might be fatal if not recognised and treated.[3]

CAH patients are generally treated with glucocorticoids and, if necessary, also with mineralocorticoids to compensate for their deficiencies. In addition, glucocorticoid treatment leads to a reduction of adrenal androgens due to a decrease in ACTH levels by restoring the negative feedback on the pituitary gland. Thus, undertreatment with suboptimal androgen suppression results in signs of androgen excess as mentioned above. Whereas overtreatment causes negative effects on bone metabolism and the cardiovascular system.[3] Therefore, treatment should be carefully balanced and monitored by measuring the typical CAH markers, A4 and 17-OHP.[4] It is recommended that A4 levels should be normalised to age- and sex-specific reference values, whereas normal or suppressed 17-OHP levels indicate overtreatment.[5]

In serum, most steroid molecules are considered biologically inactive when they are bound to proteins. Free (i.e., unbound) steroid hormones can bind their receptor and are, therefore, biologically active. Salivary hormone concentrations most likely only reflect this free portion of steroids.[6] Interestingly, steroid concentrations are not just a dialysate of serum as steroids are exposed to enzymes in the salivary gland. For instance, 11-hydroxysteroid dehydrogenase activity in the salivary glands converts cortisol into cortisone, which lowers the salivary cortisol concentration.[6]

For A4 and 17-OHP, there is a very strong correlation between steroid concentrations in serum and saliva.[4,7,8] Hence, measuring steroids in saliva instead of serum is an interesting alternative for monitoring CAH patients. Saliva is easy to retrieve via a noninvasive method, and presuming stability of these steroids in saliva, collection in the home situation is feasible. To interpret the salivary steroid concentrations of both A4 and 17-OHP, well-established reference values in healthy volunteers are essential for optimisation treatment in CAH patients. Establishing these reference values is challenging considering the diurnal rhythm and age-dependency of these steroid levels.[9] Current guidelines recommend glucocorticoid dosages three times a day.[3] Therefore, measuring A4 and 17-OHP at several time points during the day is useful to optimise treatment,[10,11] underlining the additional advantage of the possibility of at-home saliva collection.

Steroid measurement in serum or saliva is preferably performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[5] As A4 and 17-OHP are present at very low concentrations in saliva compared to serum, a sensitive measurement method by LC-MS/MS is necessary.

Here, we developed an LC-MS/MS method for measuring salivary A4 and 17-OHP concentrations, investigated their stability at ambient temperature, described the diurnal variation in healthy paediatric and adult individuals, and established reference values for A4 and 17-OHP in saliva to monitor CAH patients during treatment.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

In total, 255 volunteers participated in this study, who collected at least 2 ml saliva in the morning (7:00–8:00 AM), afternoon (15:00–16:00), and evening (22:00–23:00 or bedtime). All samples were collected after rising up (morning) or before going to bed (evening). We measured hormone concentrations at timepoints where CAH patients usually take their hydrocortisone medication. To adjust the dosage of the medication for each individual time point, A4 and 17-OHP are commonly measured just before taking the hydrocortisone medication. Among the volunteers were healthy children from two Dutch elementary schools, their parents, and other adult volunteers (4–75 years old). Ethical approval was obtained at the local medical ethics committee (Radboudumc; case number: 2014–1323). Samples were collected without the use of any additional stimulation of saliva production by directly drooling into a 15 ml polypropylene Flacon tube (BD Biosciences). Participants collected morning samples in a fasting state and before brushing teeth or smoking. Exclusion criteria were the use of glucocorticoids, pregnancy, children under 4 years of age, or working night shifts. Participants were asked to fill in their birth year and month, sex, menopausal status (for women), use of medication (including contraceptives), and exact time of saliva collection. Saliva was collected in polypropylene tubes and samples were retrieved at the laboratory by mail within 2 days after collection. Samples were frozen at −20°C until analysis by LC-MS/MS. All samples were measured in duplicate. Results below the lower limit of quantification (LLOQ) were assigned a value of half the LLOQ.

### 2.2 | Sample preparation and LC-MS/MS method

A4 (androst-4-ene-3,17-dione) and 17-OHP (17α-hydroxypregn-4-ene-3,20-dione) were analysed by LC-MS/MS after solid-phase extraction. A 9-point calibration series was prepared (Steraloids), aliquoted, and

stored at −40°C until analysis. In each run, one aliquot per calibration point was used. In-house saliva quality controls and samples were measured in duplicate. Saliva was sonicated (130 W, 20 kHz, amplitude 30%; Sonics and Materials Inc.) for 10 s at room temperature and internal standards [$^{13}C_3$]-A4 (4.8 nmol/l final concentration) and [$^{13}C_3$]-17-OHP (5.1 nmol/l final concentration) (both from IsoSciences) were added to 500 µl samples with subsequent solid-phase extraction using Oasis® MCX 1cc cartridges (Waters Corporation). Columns were pre-equilibrated with 1 ml methanol:isopropanol (95:5) and subsequently washed with 1 ml $H_2O$. After the application of the sample, columns were washed with 1 ml $H_2O$:$NH_4OH$ (95:5) and 1 ml methanol:$H_2O$:-formic acid (20:78:2). Afterward, 300 µl eluate (methanol) was dried under a stream of $N_2$ gas, reconstituted in methanol:$H_2O$ (30:70), and injected (10 µl) into an Agilent Technologies 1290 Infinity VL UHPLC-system (Agilent Technologies) equipped with a BEH C18 (1.7 µm 2.1 × 50 mm) analytical column (Waters Corporation) at 60°C. Mobile phase A (methanol:$H_2O$ 20:80 + 2 mM $NH_4CH_3COO$ + 0.1% formic acid) and B (methanol:$H_2O$ 98:2 + 2 mM $NH_4CH_3COO$ + 0.1% formic acid) were run in a gradient (0.4 ml/min). The gradient program was as follows: start gradient 70:30 A:B for 2.5 min; then to 60:40 A:B in 2 min; followed by a gradient in 2.5 min to 35:65 and a subsequent gradient in 0.5 min to 2:98 to remain for 0.5 min and thereafter to 70:30 A:B in 0.5 min and remain for 0.5 min. The retention time was 3.6 and 5.0 min, for A4 and 17-OHP, respectively, with a total run time of 9 min.

An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in electrospray positive ion mode, with a capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350°C, and gas temperature 150°C with $N_2$ collision gas. The collision energy was optimised for all analytes. Two mass transitions were monitored for each analyte and their internal standards. The first transition was used for quantification, the second for confirmation. Transitions Q1 > Q3 and corresponding collision energy (between brackets) were *m/z* 287 > 109 (26 electron volt [eV]) and *m/z* 287 > 97 (23 eV) for A4; *m/z* 290 > 112 (26 eV) and *m/z* 290 > 100 (21 eV) for [$^{13}C_3$]-A4; *m/z* 331 > 109 (31 eV) and *m/z* 331 > 97 (31 eV) for 17-OHP; and *m/z* 334 > 112 (33 eV) and *m/z* 334 > 100 (30 eV) for [$^{13}C_3$]-17-OHP. Dwell time was 50 ms for both A4 and 17-OHP.

## 2.3 | Method validation

We assessed linearity by CLSI EP6 protocol[12] in saliva. Ion suppression was assessed by continuous infusion of the labelled steroid. We assessed imprecision by adapted CLSI EP5 protocol[13] with pooled saliva samples (*n* = 8) at 184 pmol/L and 882 pmol/L for A4 and 17-OHP, respectively; the LLOQ was assessed from pooled saliva at the estimated concentration by between-day assay repeated measurements (*n* = 6) and defined as lowest value with an interassay CV of 20% (A4) or 10% (17-OHP). Recovery was calculated in samples containing 300 pmol/L of A4 and 30 pmol/L of 17-OHP to which 180 and 5000 pmol/L A4 and 110 and 6000 pmol/L 17-OHP were added.

## 2.4 | Stability of steroids in saliva

To evaluate the stability of steroids in saliva, saliva samples with a wide range of A4 and 17-OHP concentrations from 11 patients were split into 4 portions. One portion was immediately frozen at −20°C. Other portions were kept at room temperature for 24, 72, and 120 h whereafter they were frozen at −20°C. Three days after freezing the last samples, all samples were analysed in the same batch.

## 2.5 | Data analysis and statistics

Dunn's multiple comparison test was used to assess significance between sexes in GraphPad Prism statistical software. We assessed the significance between the different time points during the day by using a linear mixed models analysis in R statistical software (version 3.6.3) and included participant number and time of saliva collection as a random effect. Dixons criteria were used for outlier identification (Q > 0.414).[14] Smoothing reference centile curves were obtained with a method developed by Cole and Green.[15] This method generates LMS parameters (Lambda for the skew, Mu for the median, and Sigma for the generalised coefficient of variation) that are used to calculate *z*-scores. Salivary steroid reference values were calculated by a transformed parametric method with EP evaluator v11 statistical software.

# 3 | RESULTS

## 3.1 | LC-MS/MS method validation

The LC-MS/MS measurement method was developed for clinical use under ISO15189 accreditation. The method was linear at concentrations between 110 and 24,700 pmol/l for A4 and 14–26,800 pmol/l for 17-OHP with less than 0.6% deviation from linearity. Imprecision, LLOQ, ion suppression, and recovery after addition are shown in Table 1.

## 3.2 | Stability of A4 and 17-OHP in human saliva

The stability of the steroids in saliva was investigated to ensure that these steroids in samples obtained in the study were not degraded during transport to the laboratory. A4 and 17-OHP (*n* = 11) concentrations remained stable (<10% deviation) up to 120 h at ambient temperature before storage at −20°C (Figure S1).

## 3.3 | Diurnal rhythm and age-dependency of salivary A4 and 17-OHP

In total, 255 volunteers collected saliva, of whom four were excluded before analysis (no questionnaire filled in (*n* = 1); pregnancy

**TABLE 1** Imprecision (within and between day), LLOQ, ion suppression, and recovery of A4 and 17-OHP by LC-MS/MS

| Steroid | Imprecision (within-day) | Imprecision (between days) | LLOQ (pmol/L) | Ion suppression | Recovery |
|---|---|---|---|---|---|
| A4 | 2.3% | 2.9% | 7 | 13% | 100%–103% |
| 17-OHP | 2.5% | 4.3% | 20 | 14% | 99%–101% |

Abbreviations: 17-OHP, 17-hydroxyprogesterone; A4, androstenedione; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification.

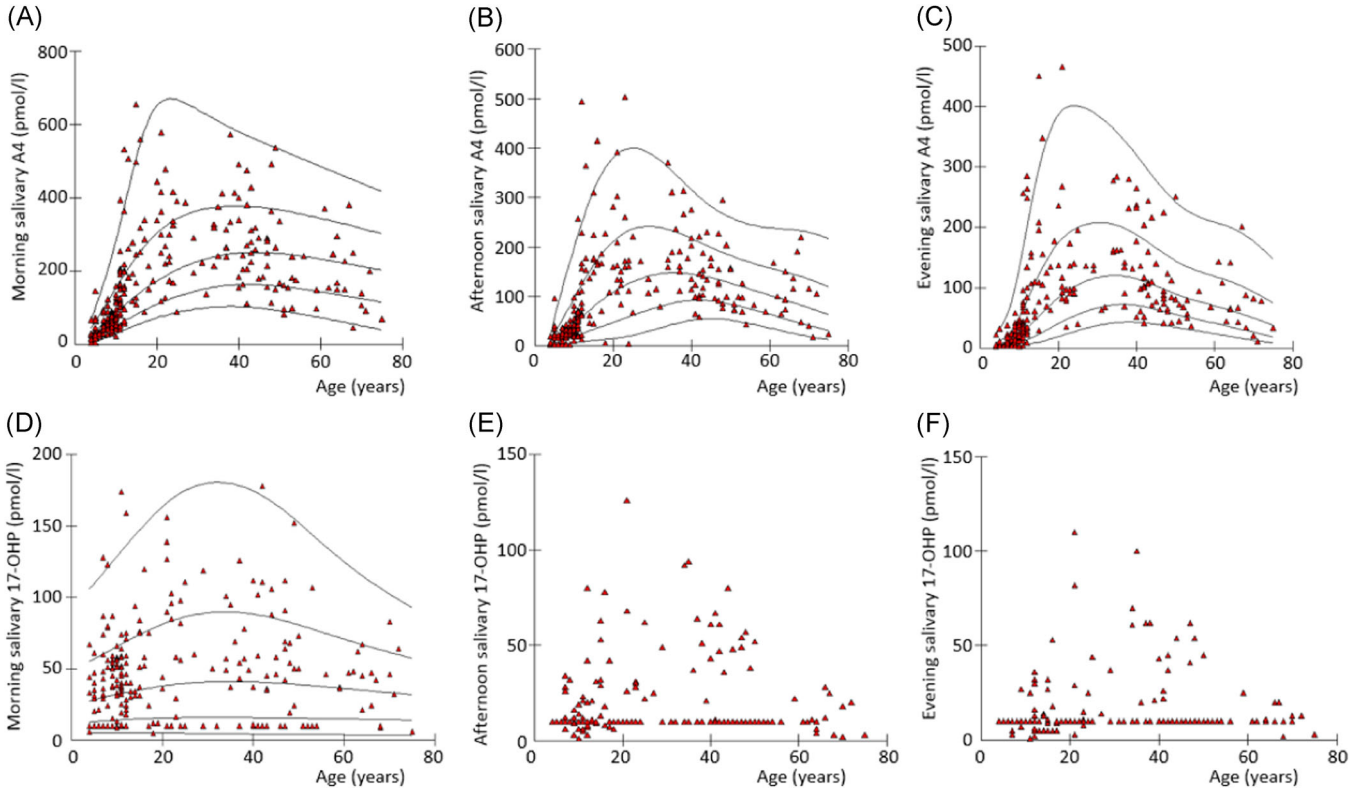


**FIGURE 1** Individual data points for salivary A4 and 17-OHP concentrations with fitted percentile lines (2.5; 17; 50; 83; 97.5 percentiles) plotted against age. No percentile curves could be generated using the LMS method for afternoon and evening 17-OHP levels. (A) Morning A4 levels ($n = 249$); (B) afternoon A4 levels ($n = 244$); (C) evening A4 levels ($n = 250$); (D) morning 17-OHP levels ($n = 247$); (E) afternoon 17-OHP levels ($n = 237$); and (F) evening 17-OHP levels ($n = 244$). 17-OHP, 17-hydroxyprogesterone; A4, androstenedione

($n = 1$); aberrant measured cortisol >4SDS ($n = 1$); and year of birth unknown ($n = 1$)). The remaining 251 volunteers had an age range of 4–75 years. Saliva samples were collected at various time points during the day (morning, afternoon, and evening) to quantify hormone concentrations over the day. There was no significant difference in A4 nor 17-OHP between males and females. An age-dependent trend was observed for A4 and 17OHP levels with increasing levels of both A4 and 17OHP up to the age of approximately 30 years, after which this rise stagnates and decreases after the age of approximately 40 years (Figure 1). Median A4 levels were 149 pmol/l (interquartile range [IQR]: 66–251), 71 pmol/l (IQR: 30–153), and 49 pmol/l (IQR: 13–106), for the morning, afternoon, and evening, respectively. Median morning 17-OHP levels were 40 pmol/l (IQR: 10–59). For the afternoon and evening, median 17-OHP levels were below the LLOQ. Concentrations of both steroids decreased significantly during the day in comparison to the morning concentrations ($p < .001$).

Percentile curves (2.5; 17; 50; 83; 97.5) were estimated and represent the −2; −1; 0; +1; +2SD, respectively. Percentile curves for afternoon and evening 17-OHP levels could not be generated.

## 3.4 | Establishment of reference values for salivary A4 and 17-OHP

For the calculation of reference values, we selected two different cohorts (children and adults) from the data described above.

Reference values were calculated for children (4–9 years for girls and 4–10 years for boys) and adults (16–51 years). As we were not able to assess pubertal stages in the children, girls between 10 and 15

years of age ($n = 36$) and boys between 11 and 15 years of age ($n = 20$) were excluded from the paediatric cohort, since variable pubertal stages in this age groups might influence the reference values. According to age, it is assumed that the paediatric cohort represents reference values of prepubertal children.[16–19] The paediatric reference cohort consisted of 80 children (40 boys, 40 girls) with a median age of 8 years (range 4–10).

Adult volunteers (16–51 years) using steroid hormone medication (including contraceptives) ($n = 26$), postmenopausal women ($n = 18$), premenarchal girls ($n = 1$), and men above 51 years of age ($n = 12$) were excluded from the reference population to ensure an unequivocal cohort. The adult reference group consisted of 59 volunteers (33 males, 26 females) with a median age of 37 years (range 16–51). The mean time points (and standard deviation) of sample collection for the paediatric age group were 7:28 (0:19), 15:13 (0:42), and 19:50 (0:45) for the morning, afternoon, and evening, respectively. For the adult reference population mean time points (and standard deviations) were 7:38 (0:46), 15:30 (0:46), and 21:51 (2:58) for the morning, afternoon, and evening, respectively.

The resulting time-dependent reference values for salivary A4 and 17-OHP are shown in Table 2. Whenever the calculated reference limit was below the LLOQ this is denoted as "<LLOQ." For A4 both upper and lower reference limits are given since the treatment goal for CAH patients is to aim for A4 values within these limits. For 17-OHP, only the upper limit values are clinically relevant for monitoring CAH patients, since 17-OHP levels below this upper reference limit indicate overtreatment.[5] Lower limit reference values were below LLOQ (<20 pmol/L) and therefore, only 17-OHP upper limit values are given in Table 2.

## 4 | DISCUSSION

In this study, we quantified A4 and 17-OHP concentrations in the morning, afternoon, and evening and demonstrated that there is a significant rhythm with the highest levels in the morning and decreasing levels over the day. We demonstrated age-dependency of A4 and 17-OHP concentrations in saliva and established separate reference values at these time points for both children and adults. To the best of our knowledge, this is the first study that quantified salivary diurnal A4 and 17-OHP levels in both healthy children and adults by LC-MS/MS in a large study cohort. These values may aid in monitoring CAH patients during treatment.

To allow monitoring of CAH patients on short-acting glucocorticoids, we quantified A4 and 17-OHP concentrations at three-time points during the day. In addition, these reference values can be used to monitor CAH patients on long-acting glucocorticoids but in these patients, pharmacodynamics can be different. Therefore, it is important to consider the type of glucocorticoid and time of administration when interpreting the salivary A4 and 17-OHP concentration. In the early morning, A4 and 17-OHP are at their highest levels and decrease during the day, which is consistent with a diurnal rhythm seen in previous studies.[8,9,20] This rhythm emphasises the importance of time-controlled sample collection and time-specific reference values for monitoring CAH patients during the day.

The salivary concentration of A4 and 17-OHP is more than 10-fold lower compared to serum values. In serum, these steroids are to a large extent bound to proteins. Presumably, salivary steroid concentrations only reflect the free (nonprotein bound) fraction, resulting in lower concentrations. Salivary steroid concentrations are not only a dialysate of these free serum concentrations as steroid hormones might also be subject to steroid conversion in the salivary gland, as seen with cortisol and cortisone.[6]

We observed considerably higher salivary A4 and 17-OHP concentrations in adults compared to prepubertal children, especially for A4, consistent with earlier findings in serum.[21–25] In addition to the adrenal glands, the gonads of both adult men and women produce A4 and 17-OHP as precursors in the pathway to oestrogens and testosterone. In healthy women, approximately half of the A4 present in serum is produced by the ovaries.[26] The rise in salivary A4 and 17-OHP during puberty observed in our study is most likely due to the activation of the gonadostat. After the age of 40 years, there is a decrease in salivary A4 and 17-OHP levels, which is consistent with previous studies in serum.[25,27] Stanczyk showed that postmenopausal serum A4 levels are lower compared to the concentration of premenopausal women, probably due to declining ovary function.[25] In males the decrease in the steroid concentration is most likely due to declining Leydig cell function and increasing concentrations of sex-hormone-binding globulin with increasing age.[27]

Glucocorticoid treatment in CAH patients is most often given three times a day.[3] To facilitate easier monitoring of treatment of CAH patients in clinical practice and adjust dosage at individual time points, analysis of A4 and 17-OHP in saliva collected at these time points is most informative.[5] Preferably patients collect the saliva at home and send samples to the laboratory. To enable the home collection of saliva, A4 and 17-OHP concentrations must be stable during transport.

**TABLE 2** Reference intervals for A4 and upper reference limits for 17-OHP were established in healthy children (girls 4–9 years old; boys 4–10 years old) and adults (16–51 years old)

| Steroid | Group | Reference values (pmol/l) | | |
|---|---|---|---|---|
| | | Morning | Afternoon | Evening |
| A4 | Children | 10–123 ($n = 80$) | <7–54 ($n = 77$) | <7–42 ($n = 80$) |
| | Adults | 119–553 ($n = 57$) | 40–363 ($n = 57$) | 42–319 ($n = 59$) |
| 17-OHP | Children | <110 ($n = 78$) | <22 ($n = 76$) | <20 ($n = 78$) |
| | Adults | <170 ($n = 57$) | <110 ($n = 56$) | <67 ($n = 58$) |

Abbreviations: 17-OHP, 17-hydroxyprogesterone; A4, androstenedione.

We demonstrated good stability of A4 and 17-OHP concentrations in saliva at room temperature up to 5 days after collection, which makes noninvasive salivary steroid measurements an interesting alternative, especially in a paediatric population. The marginal decrease in 17-OHP after 5 days found in our study is consistent with an earlier report where an approximately 10% decrease in 17-OHP was found at room temperature after 5 days.[28] To the best of our knowledge, stability experiments for A4 in saliva have not been reported before. As this androgen is indispensable for monitoring CAH patients, demonstrating stability is crucial for at-home collecting of saliva for this purpose.

Our results are comparable to saliva steroid concentrations found in healthy individuals in other studies,[7,29,30] demonstrating their validity across multiple independent LC-MS/MS methods. Turpeinen et al.[31] only reported reference values for A4 in the morning. Our early morning A4 reference values are higher compared to values in their study, probably due to the earlier time point of sample collection in our study (7:00–8:00 AM vs. 7:00–9:00 AM).[31]

In previous studies,[4,9] salivary A4 and 17-OHP concentrations were measured in healthy children using an immunoassay, and they reported higher values compared to our results. A positive bias of immunoassays compared to LC-MS/MS methods has been found more often and may be due to cross-reactivity with other steroids.[31,32]

In our study, we did not observe a sex-specific difference in salivary A4 nor 17-OHP concentrations in prepubertal children nor in adults for all individual time points during the day. For prepubertal children, this finding is consistent with other reports on salivary steroid measurements,[9,33] and most likely due to the limited contribution of gonadal steroid production in prepubertal children. Turpeinen et al.[31] reported comparable upper reference limits for salivary A4 levels between males and females in their cohort of healthy adults, confirming the absence of sex-specific differences as seen in our study. In serum, sex-specific differences for both A4 and 17-OHP levels have been reported.[21] However, these differences were subtle (males 1.53–8.28 nmol/l vs. females 1.06–7.72 nmol/l for A4; 1.87–6.24 nmol/l vs. 1.06–6.84 nmol/l for 17-OHP). Interestingly, in their study more than half of the female reference cohort consisted of postmenopausal women, which presumably results in lower reported female serum reference values. In our saliva study, only males and premenopausal women between 16 and 51 years of age were included to calculate reference values. Moreover, Eisenhofer et al.[21] observed higher A4 levels during the luteal phase compared to the follicular phase, even exceeding male serum A4 levels.

Our study has several strengths. First, samples were collected in the morning, afternoon, and evening, which gives insight into the diurnal variation and enables the establishment of reference values for various time points during the day. Second, considering the age-dependency, saliva was collected from volunteers in a broad age range, and reference values for both children and adults were established. Finally, all steroid measurements were performed using LC-MS/MS, which is the gold standard for (salivary) quantification of steroid concentrations.[5]

A limitation of this study is that we did not establish reference values for girls between 10 and 15 years and boys between 11 and 15 years old, as the various pubertal stages in this age group were not assessed for necessary subdivision. In addition, it is known that salivary 17-OHP levels fluctuate during the menstrual cycle in both healthy individuals and CAH patients.[34,35] As we did not take the menstrual phases into account, this might have led to a larger variation in A4 and 17-OHP concentrations within the female cohort. The fluctuations during the menstrual cycle are due to ovarian A4 and 17-OHP production.[34] Reported variations are limited in respect to the A4 and 17-OHP levels found in CAH patients[35] as in these patients the adrenal glands are the predominant source of A4 and 17-OHP. Therefore, these minor fluctuations due to ovarian production are not relevant for clinical decision-making in CAH patients.

Besides monitoring CAH patients, salivary steroid measurements might eventually also be useful in the diagnosis and/or treatment monitoring of other diseases with altered steroid concentrations. However, more research is necessary to elucidate salivary steroid levels in these diseases. We recommend considering the time point of saliva collection in interpreting the laboratory results.

In conclusion, we established an LC-MS/MS method for measuring A4 and 17-OHP levels in saliva and demonstrated diurnal variation and age-dependency in healthy volunteers, including children and adults. In addition, we established reference values for the paediatric and adult population at three-time points during the day. The reference values support treatment monitoring of children and adults with CAH.

## ACKNOWLEDGEMENTS

The authors would like to thank Ben J. Pelzer for his assistance in the statistical analysis, and Trudy van Herwaarden and Cleo van Herwaarden for distributing the supplies needed for saliva collection.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data will be made available in a repository if this article is accepted for publication.

## ORCID

*Bas P. H. Adriaansen* http://orcid.org/0000-0003-1439-236X
*Mariska A. M. Schröder* http://orcid.org/0000-0003-2139-9076

## REFERENCES

1. El-Maouche D, Arlt W, Merke DP. Congenital adrenal hyperplasia. *Lancet*. 2017;390(10108):2194-2210.
2. Engels M, Span PN, van Herwaarden AE, Sweep F, Stikkelbroeck N, Claahsen-van der Grinten HL. Testicular adrenal rest tumors: current insights on prevalence, characteristics, origin, and treatment. *Endocr Rev*. 2019;40(4):973-987.
3. Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, et al. Congenital adrenal hyperplasia–current insights in pathophysiology, diagnostics and management. *Endocr Rev*. 2022;43:91-159.
4. Otten BJ, Wellen JJ, Rijken JC, Stoelinga GB, Benraad TJ. Salivary and plasma androstenedione and 17-hydroxyprogesterone levels in congenital adrenal hyperplasia. *J Clin Endocrinol Metab*. 1983;57(6):1150-1154.
5. Speiser PW, Arlt W, Auchus RJ, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab*. 2018;103(11):4043-4088.
6. Blair J, Adaway J, Keevil B, Ross R. Salivary cortisol and cortisone in the clinical setting. *Curr Opin Endocrinol Diabetes Obes*. 2017;24(3):161-168.

7. Bacila I, Adaway J, Hawley J, et al. Measurement of salivary adrenal-specific androgens as biomarkers of therapy control in 21-hydroxylase deficiency. *J Clin Endocrinol Metab*. 2019;104(12):6417-6429.
8. Mezzullo M, Fazzini A, Gambineri A, et al. Parallel diurnal fluctuation of testosterone, androstenedione, dehydroepiandrosterone and 17OHprogesterone as assessed in serum and saliva: validation of a novel liquid chromatography-tandem mass spectrometry method for salivary steroid profiling. *Clin Chem Lab Med*. 2017;55(9):1315-1323.
9. Gröschl M, Rauh M, Dörr HG. Circadian rhythm of salivary cortisol, 17alpha-hydroxyprogesterone, and progesterone in healthy children. *Clin Chem*. 2003;49(10):1688-1691.
10. Birkebaek NH, Hougaard DM, Cohen AS. Monitoring steroid replacement therapy in children with congenital adrenal hyperplasia. *J Pediatr Endocrinol Metab*. 2017;30(1):85-88.
11. Al-Kofahi M, Ahmed MA, Jaber MM, et al. An integrated PK-PD model for cortisol and the 17-hydroxyprogesterone and androstenedione biomarkers in children with congenital adrenal hyperplasia. *Br J Clin Pharmacol*. 2021;87(3):1098-1110.
12. McEnroe RJ, Paul Durham A, Kondratovich MV, et al. Evaluation of Linearity of Quantitative Measurement Procedures, *in* EP06. Clinical and Laboratory Standards Institute; 2020.
13. McEnroe RJ. *Evaluation of Precision of Quantitative Measurement Procedures EP05*. 3 ed. Clinical and Laboratory Standards Institute; 2014.
14. Rorabacher DB. Statistical treatment for rejection of deviant values: critical values of Dixon's "Q" parameter and related subrange ratios at the 95% confidence level. *Anal Chem*. 1991;63(2):139-146.
15. Cole TJ, Green PJ. Smoothing reference centile curves: the LMS method and penalized likelihood. *Stat Med*. 1992;11(10):1305-1319.
16. Fredriks AM, van Buuren S, Burgmeijer RJ, et al. Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res*. 2000;47(3):316-323.
17. Mul D, Fredriks AM, van Buuren S, Oostdijk W, Verloove-Vanhorick SP, Wit JM. Pubertal development in The Netherlands 1965-1997. *Pediatr Res*. 2001;50(4):479-486.
18. Joustra SD, van der Plas EM, Goede J, et al. New reference charts for testicular volume in Dutch children and adolescents allow the calculation of standard deviation scores. *Acta Paediatr*. 2015;104(6):e271-e278.
19. Talma H, Schönbeck Y, van Dommelen P, Bakker B, van Buuren S, Hirasing RA. Trends in menarcheal age between 1955 and 2009 in the Netherlands. *PLoS One*. 2013;8(4):e60056.
20. Young MC, Walker RF, Riad-Fahmy D, Hughes IA. Androstenedione rhythms in saliva in congenital adrenal hyperplasia. *Arch Dis Child*. 1988;63(6):624-628.
21. Eisenhofer G, Peitzsch M, Kaden D, et al. Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: impact of gender, age, oral contraceptives, body mass index and blood pressure status. *Clin Chim Acta*. 2017;470:115-124.
22. Kushnir MM, Blamires T, Rockwood AL, et al. Liquid chromatography-tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clin Chem*. 2010;56(7):1138-1147.
23. Haring R, Hannemann A, John U, et al. Age-specific reference ranges for serum testosterone and androstenedione concentrations in women measured by liquid chromatography-tandem mass spectrometry. *J Clin Endocrinol Metab*. 2012;97(2):408-415.
24. Søeborg T, Frederiksen H, Mouritsen A, et al. Sex, age, pubertal development and use of oral contraceptives in relation to serum concentrations of DHEA, DHEAS, 17alpha-hydroxyprogesterone, Delta4-androstenedione, testosterone and their ratios in children, adolescents and young adults. *Clin Chim Acta*. 2014;437:6-13.
25. Stanczyk FZ. Measurement of androgens in women. *Semin Reprod Med*. 2006;24(2):78-85.
26. Burger HG. Androgen production in women. *Fertil Steril*. 2002;77:3-5.
27. Gray A, Feldman HA, McKinlay JB, Longcope C. Age, disease, and changing sex hormone levels in middle-aged men: results of the Massachusetts male aging study. *J Clin Endocrinol Metab*. 1991;73(5):1016-1025.
28. Gröschl M. Stability of salivary steroids: the influences of storage, food and dental care. *Steroids*. 2001;66(10):737-741.
29. McHale TS, Gray PB, Chan KC, Zava DT, Chee WC. Salivary steroid hormone responses to dyadic table tennis competitions among Hong Kongese juvenile boys. *Am J Hum Biol*. 2018;30(6):e23190.
30. Büttler RM, Bagci E, Brand HS, den Heijer M, Blankenstein MA, Heijboer AC. Testosterone, androstenedione, cortisol and cortisone levels in human unstimulated, stimulated and parotid saliva. *Steroids*. 2018;138:26-34.
31. Turpeinen U, Hämäläinen E, Haanpää M, Dunkel L. Determination of salivary testosterone and androstendione by liquid chromatography-tandem mass spectrometry. *Clin Chim Acta*. 2012;413(5-6):594-599.
32. Fanelli F, Belluomo I, Di Lallo VD, et al. Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults. *Steroids*. 2011;76(3):244-253.
33. de Groot MJ, Pijnenburg-Kleizen KJ, Thomas CMG, et al. Salivary morning androstenedione and 17alpha-OH progesterone levels in childhood and puberty in patients with classic congenital adrenal hyperplasia. *Clin Chem Lab Med*. 2015;53(3):461-468.
34. Gröschl M, Rauh M, Schmid P, Dörr H-G. Relationship between salivary progesterone, 17-hydroxyprogesterone, and cortisol levels throughout the normal menstrual cycle of healthy postmenarcheal girls. *Fertil Steril*. 2001;76(3):615-617.
35. Stikkelbroeck NMML, Sweep CGJ, Braat DDM, Hermus ARMM, Otten BJ. Monitoring of menstrual cycles, ovulation, and adrenal suppression by saliva sampling in female patients with 21-hydroxylase deficiency. *Fertil Steril*. 2003;80(4):1030-1036.


## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Adriaansen BPH, Kamphuis JS, Schröder MAM, et al. Diurnal salivary androstenedione and 17-hydroxyprogesterone levels in healthy volunteers for monitoring treatment efficacy of patients with congenital adrenal hyperplasia. *Clin Endocrinol (Oxf)*. 2022;97:36-42. doi:10.1111/cen.14690