Clinical Chemistry 56:7
1138–1147 (2010)

Liquid Chromatography–Tandem Mass Spectrometry Assay for Androstenedione, Dehydroepiandrosterone, and Testosterone with Pediatric and Adult Reference Intervals

Mark M. Kushnir,[1,2*] Takara Blamires,[1] Alan L. Rockwood,[1,2] William L. Roberts,[1,2] Bingfang Yue,[1] Evrim Erdogan,[2] Ashley M. Bunker,[1] and A. Wayne Meikle[1,2,3]

BACKGROUND: Measurement of serum androgens is important in adult, geriatric, pediatric endocrinology, and oncology patients. We developed a liquid chromatography—tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of androstenedione, dehydroepiandrosterone (DHEA), and testosterone in these patients.

METHODS: We spiked 200 μL of serum or plasma with isotope-labeled internal standards and performed extraction with methyl t-butyl ether. We then derivatized the extracts with hydroxylamine and analyzed them by LC-MS/MS using a 2-dimensional chromatographic separation with a 3.5-min analysis time.

RESULTS: Total imprecision for each analyte was <11.2%. Limits of quantification were 10, 50, and 10 ng/L for androstenedione, DHEA, and testosterone, respectively. Reference intervals were established for children (age 6 months to 17 years), men, and women. Androstenedione and DHEA concentrations were lowest in 2- to 3-year-old children. Adult concentrations were achieved in girls at Tanner stage 3 and in boys at Tanner stage 4–5. In premenopausal and (postmenopausal) women the median concentrations of androstenedione, DHEA, and testosterone were 810 (360), 3000 (1670), 270 (180) ng/L, respectively. In postmenopausal women, concentrations of testosterone were age independent, whereas androstenedione and DHEA concentrations decreased with age. In men the median concentrations of androstenedione, DHEA, and testosterone were 440, 2000, and 3700 ng/L, respectively. In men older than 40 years, median concentrations decreased at rates of 5%, 10%, and 20% per decade for androstenedione, DHEA, and testosterone, respectively.

CONCLUSIONS: This LC-MS/MS method has the required lower limit of quantification and specificity for analysis of endogenous concentrations of androgens in all groups studied. Reference intervals were established for healthy children and adults.

Androstenedione and dehydroepiandrosterone (DHEA)[4] are androgen precursors of male and female sex hormones; testosterone and dihydrotestosterone are bioactive androgens responsible for development of male-specific characteristics and many non–sex-specific functions *(1)*. Androstenedione is produced in large amounts by both the adrenal glands and gonads, and DHEA is primarily produced by the adrenal glands. In males, the testes secrete approximately 95% of testosterone and the rest is made from adrenal androgen precursors by the peripheral conversion (in liver, skin, and adipose tissue) of androstenedione. In females, ovaries and adrenal-gland androgen precursors result in approximately 50% of testosterone, and the rest is produced by the peripheral conversion of androstenedione. Androgens are responsible for the development and maintenance of masculine characteristics and serve as estrogen precursors. The main pathway of androgen biosynthesis is through the conversion of 17-hydroxypregnenolone to DHEA (controlled by cytochrome P17), DHEA to androstenedione [controlled by 3β hydroxysteroid dehydrogenase (3βHSD)], androstenedione to estrone, and estrone and testosterone to estradiol (both controlled by cytochrome P19).

Measurement of testosterone has many clinical applications. In men, testosterone is usually measured to evaluate hypogonadism. In women, it is measured in

[1] ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; [2] Department of Pathology, University of Utah, Salt Lake City, UT; [3] Departments of Medicine and Pathology, University of Utah, Salt Lake City, UT.
* Address correspondence to this author at: ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. Fax 801-584-5207; e-mail kushnmm@aruplab.com.

Received January 7, 2010; accepted April 20, 2010.
Previously published online at DOI: 10.1373/clinchem.2010.143222

[4] Nonstandard abbreviations: DHEA, dehydroepiandrosterone; 3βHSD, 3β hydroxysteroid dehydrogenase; DHT, dihydrotestosterone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; TS, Tanner stage.

the investigation of hyperandrogenism, hirsutism, virilization, oligo/amenorrhea, acne, and infertility. Increased total and free testosterone concentrations can arise from excessive adrenal steroid synthesis, tumors of adrenal origin, and polycystic ovarian syndrome *(2–5)*. In children, testosterone is measured for sex assignment in infants with ambiguous genitalia, for diagnosis of disorders of puberty, and in the follow-up of patients with congenital adrenal hyperplasia *(6–10)*.

Excessive production of androstenedione can be caused by defects of adrenal steroid biosynthesis, tumors of ovarian and adrenal origin, polycystic ovarian syndrome, increased peripheral sensitivity to androgens, and increased peripheral production of androgens *(1, 3–6)*. DHEA is used as a marker of adrenal androgen production; abnormally low concentrations of DHEA may occur in patients with adrenal insufficiency, older adults, and peri- and postmenopausal women. Increased concentrations occur in several conditions, including virilizing adrenal adenoma and carcinoma, congenital adrenal hyperplasia, and hirsutism. Because the gonads produce very little DHEA, measurement of DHEA may aid in the localization of the androgen source in virilizing conditions *(1)*. Measurement of multiple androgens can be useful for the differential diagnosis of androgen-related disorders and assessment of androgen excess in women and children *(11, 12)*.

Immunoassays for androgens are known to be susceptible to analytical interferences owing to cross-reactivity with a variety of endogenous substances, and measurement results often overestimate the true concentration, especially at low concentrations characteristic of women and children *(13–17)*. The specificity of liquid chromatography–tandem mass spectrometry (LC-MS/MS) offers advantages over immunoassays *(17–21)*.

Our aims were to develop an LC-MS/MS method suitable for quantification of androgens in serum and plasma samples from both sexes across all ages, to establish reference intervals in children and adults, and to delineate trends of androgen concentration changes in both sexes from infants to older adults.

Materials and Methods

STANDARDS AND REAGENTS

Androstenedione and DHEA were purchased from Steraloids and testosterone from Sigma. Deuterium-labeled analogs of d_7-androstenedione and d_2-DHEA were purchased from CDN Isotopes, and d_3-testosterone was purchased from Sigma. Stock standards were prepared in methanol at concentration of 1 g/L. A working combined calibration standard was prepared at concentrations of 2.5 μg/L of each analyte in 1:1 methanol/water. Working combined internal standard was prepared in a 1:1 methanol/water at concentrations of 10 μg/L of d_7-androstenedione and d_2-DHEA and 2.5 μg/L of d_3-testosterone. Calibration standards were prepared in 0.1% BSA at concentrations of 50, 100, 250, 800, 1200, and 2000 ng/L. HPLC-grade water, methanol, methyl t-butyl ether, and acetonitrile were obtained from VWR. All other reagents were purchased from Sigma and were of the highest purity commercially available.

SAMPLE PREPARATION

Aliquots of 200 μL of calibrators, controls, or patient serum were transferred into polypropylene microcentrifuge tubes. To each tube we added 20 μL of the working combined internal standard. The samples were extracted with 1 mL of methyl t-butyl ether, the organic phase was transferred into a 96-well plate and evaporated under nitrogen at 50 °C, and the dried residues were redissolved for derivatization in 75 μL of hydroxylamine (0.7 mol/L) prepared in 3:7 methanol/water. The plate was vortex-mixed and incubated at 70 °C for 15 min. After the incubation, 75 μL of water was added to each well before analysis.

LC-MS/MS

The LC-MS/MS instrument was an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/Sciex) with a TurboV™ ion source operated at 600 °C. The system included 2 Agilent 1200 pumps, a column oven and a HTC PAL autosampler (LEAP Technologies) equipped with a fast wash station and 6-port switching valve. The first dimension of separation used a phenyl cartridge and the analytical separation (second dimension) was performed on a Gemini C18 column (100 × 2.0 mm, 3 μm) (Phenomenex). The injection volume was 50 μL and the oven temperature was 30 °C. For the first-dimension separation the mobile phase was a mixture of water containing 10 mmol/L formic acid and methanol containing 10 mmol/L formic acid. For the second dimension the mobile phase was a mixture of water containing 10 mmol/L formic acid and acetonitrile containing 10 mmol/L formic acid. The mobile phase for the first-dimension separation was delivered at a flow rate of 0.75 mL/min, with a linear gradient from 15% to 97% methanol between 0 and 1.5 min, followed by reequilibration to initial conditions. The mobile phase for the analytical separation was delivered at a flow rate of 0.6 mL/min with a gradient: 30% acetonitrile for 0.5 min, linear gradient to 65% acetonitrile between 0.5 and 2.8 min, step gradient to 97%, followed by reequilibration to initial conditions. The effluent from the first column was directed into the analytical column between 0.5 and 1.2 min.

The autosampler injection syringe was washed 10 times between each injection with a 4:1 methanol/wa-

ter mix containing 20 mmol/L of trifluoroacetic acid. The injection valve was washed after each injection with 1 mL of the same solvent mix. Quadrupoles Q1 and Q3 were tuned to unit resolution, and the MS conditions optimized for maximum signal intensity for each of the androgens. The instrument was operated in positive-ion mode with an ion-spray potential of 3500 V, entrance potential of 10 V, and declustering potentials of 90 V for androstenedione and testosterone, and 60 V for DHEA. Two mass transitions were monitored for each androgen and its internal standard. Primary (secondary) mass transitions of androstenedione, DHEA, and testosterone were *m/z* 317–124 (112) Da, *m/z* 304–253 (253) Da, and *m/z* 304–112 (124) Da, respectively. The same precursor/product mass transitions for quantification and assessment of specificity were acquired for DHEA (*m/z* 304–253 Da) by using different collision energies of 27 eV and 20 eV for the primary and the secondary mass transitions, respectively. Data were acquired and processed with Analyst™ 1.4.2 software. Calibration was performed with every batch of samples.

ASSAY PERFORMANCE CHARACTERISTICS

Imprecision, limit of detection (LOD), limit of quantification (LOQ), upper limit of linearity, method comparison, interference, recovery, carryover, and ion suppression were assessed. Imprecision was determined by analyzing 3 replicates per run of human plasma samples containing androgens with concentrations ranging between 50 and 2500 ng/L in 1 run per day for a period of 5 days. Linearity was evaluated with 7 samples with androgen concentrations between 800 and 45000 ng/L. The LOQ was determined by analyzing 6 samples in duplicate over 3 days containing progressively lower concentrations of androgens down to 5 ng/L. A criterion for maintaining accuracy within ±15%, imprecision (CV) <20% and a branching ratio of the mass transitions within ±30% was used to determine the upper limit of linearity and LOQ for the assay and for assessment of the specificity in patient samples *(22)*. LOD was determined as the lowest concentration at which chromatographic peaks of the androgens were present in both transitions at expected retention times with a signal-to-noise ratio >5. Absolute recovery was determined by standard addition by using 3 patient serum samples containing 300–3600 ng/L of endogenous androgens spiked with 200 ng/L and 1000 ng/L of each, androstenedione, DHEA, and testosterone. The absolute recovery was calculated as the difference between the observed and expected concentrations of the analytes. We evaluated the extraction recovery by analyzing the same 3 patient samples with internal standard added before and after the extraction. The recovery was calculated based on the concentrations observed in the samples with internal standard added before and after extraction. All experiments for the evaluation of the absolute recovery and the extraction recovery were performed in duplicate.

To examine possible interfering substances, we analyzed more than 50 steroids and steroid metabolites (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue7), and more than 20 000 patient samples. We evaluated ion suppression by injecting extracted samples with androgen concentrations below 1000 ng/L into the flow of a postcolumn infused mixture containing oxime derivatives of androstenedione, DHEA, and testosterone. A decrease in the intensity of the baseline in each mass transition was considered evidence of ion suppression *(23)*.

QUALITY CONTROL

Evaluation of the batch acceptability included system function tests, coefficient of determination of the calibration curve ($R^2 \geq 0.995$), concentrations of the androgens in the QC samples within 2 SD of historical values, and concentrations in the negative controls below the LOQ of the method. Acceptability of every injection was evaluated on the basis of the retention time (within 1% of values in the transitions of the calibration standards), intensity of the internal standard, and the ratios of the mass transitions for the analytes and the internal standards. A branching ratio of the transitions outside of ±30% limits, broadening of the chromatographic peaks, split peaks, and an increase in the background, were interpreted as potential interference.

REFERENCE INTERVALS, SPECIMEN-TYPE SUITABILITY, AND STABILITY

After we obtained informed consent from apparently healthy volunteers, we collected blood samples in serum-separator tubes (Becton Dickinson) for reference-interval studies. Samples were allowed to clot for 30 min at room temperature and centrifuged for 10 min at 2000*g*, and then the serum removed and stored at −70 °C. The sample donors were not taking prescription medications. Women on oral contraceptives or hormone replacement therapy were excluded from the study. Blood was drawn from adult participants between 8 and 10 AM; more than 90% of participants were white. The mean (median) ages of the adult volunteers were 33.9 (33.0), 52.6 (53.1), and 39.7 (38.6) years for premenopausal women (n = 104), postmenopausal women (n = 86), and men (n = 132), respectively. Blood was collected from children (n = 2517) after we obtained parental permission. Children enrolled in the study were not taking prescription medications and had no known medical conditions. Children age 7 through 17 years were enrolled in a research

setting, and their Tanner stage (TS) was determined. Children age 6 months through 6 years were enrolled before undergoing elective surgical procedures. Blood was collected from children age 6 months through 6 years after induction of anesthesia. Reference intervals were determined by using a published statistical method *(24)*.

To determine sample stability, aliquots of plasma collected from 1 individual that contained 350, 1900, and 2500 ng/L of androstenedione, DHEA, and testosterone, respectively, were stored at room temperature, in a refrigerator (4 °C), and in a freezer (−20 °C). The samples were transferred into a −70 °C freezer after 1, 3, 7, 14, 21, and 28 days of storage and analyzed in a single batch. Blood from 7 volunteers was collected in serum-separator and sodium-EDTA tubes (for plasma) for assessment of sample-type acceptability and stability. For evaluation of storage stability, these samples were stored frozen at −70 °C and analyzed before and after storage for more than 1 year. All studies with samples from human donors were approved by the institutional review board of the University of Utah.

METHOD COMPARISON

Our method was compared with LC-MS/MS methods of a commercial laboratory for measurement of androstenedione (n = 40), DHEA (n = 40), and testosterone (n = 60). The method was also compared with commercial immunoassays for androstenedione (Immulite 2000, Siemens) and DHEA (RIA; Beckman Coulter). Samples for the method comparison were deidentified patient serum and plasma samples submitted for routine clinical testing. The results were evaluated by using Deming regression *(25)*.

Results

The chromatograms of 2 multiple-reaction monitoring transitions of androstenedione, DHEA, and testosterone extracted from serum of a TS 1 girl are shown in Fig. 1. The LOD (LOQ) for androstenedione, DHEA, and testosterone were 5 (10), 10 (50), and 5 (10) ng/L, respectively. (Conversion factors from units of ng/L to pmol/L for DHEA, androstenedione, and testosterone are 3.47, 3.49, 3.47, respectively.) The assay was linear to 40 000, 10 000, and 25 000 ng/L for androstenedione, DHEA, and testosterone, respectively. Signal-to-noise ratios for 3 pg of androstenedione, DHEA, and testosterone injected onto the HPLC column (n = 5) were 73, 52, and 93, respectively. Within-run, between-run, and total imprecision data are shown in online Supplemental Table 1. The results of the Bland-Altman method comparison with commercial immunoassays and commercial LC-MS/MS methods are shown (Fig. 2; see online Supplemental Fig. 2). This method showed acceptable agreement with other LC-MS/MS assays (Deming regression slopes of 0.74, 0.99, and 0.96, for androstenedione, DHEA, and testosterone, respectively). Proportional bias observed for andro-

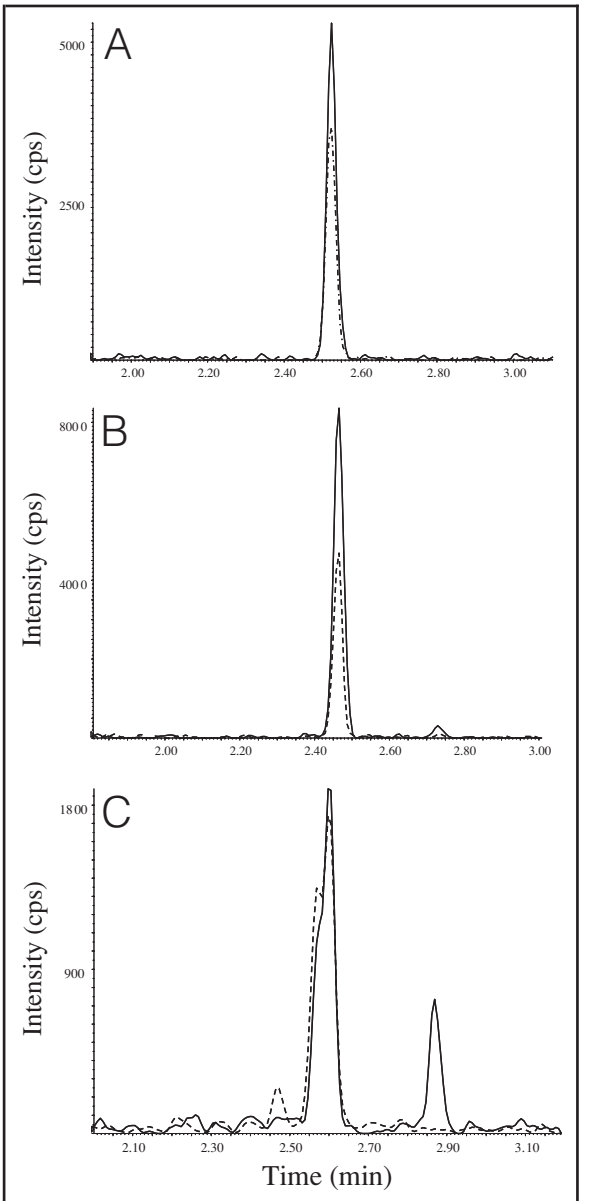

Fig. 1. Chromatograms of primary (solid lines) and secondary (dashed lines) mass transitions of androstenedione (A), DHEA (B), and testosterone (C) in a serum sample from a girl in TS 1.

The peak shape of testosterone is caused by coelution of 2 partially resolved peaks of isomers (testosterone-oxime derivatives) formed during the derivatization.

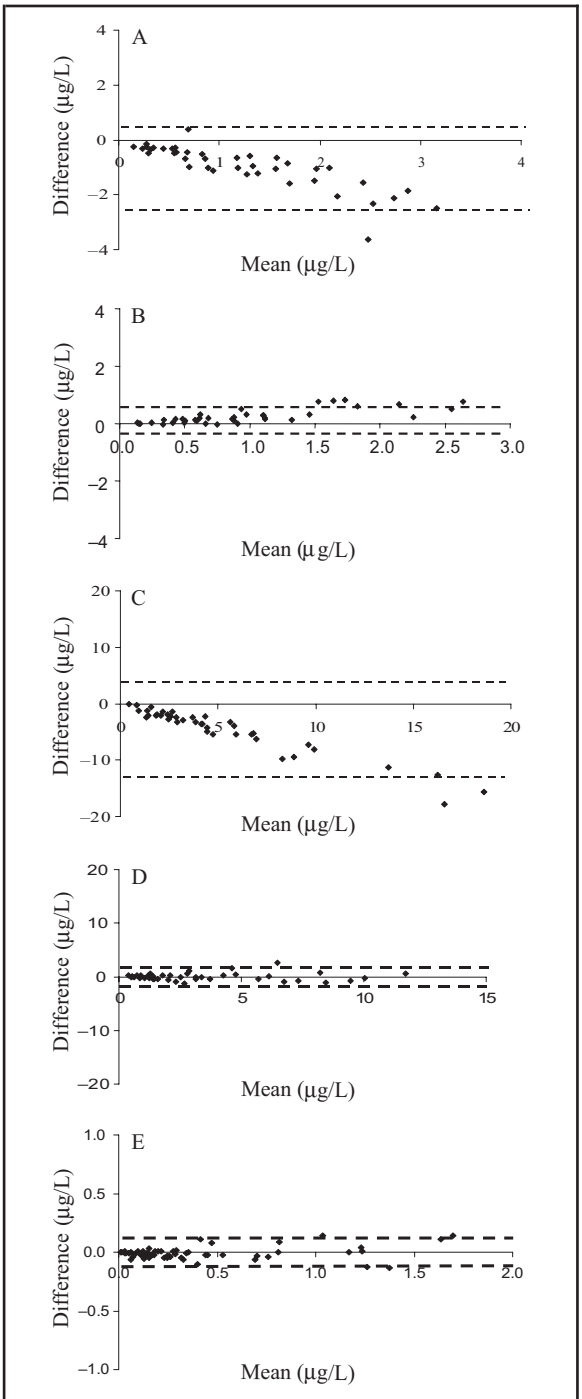

Fig. 2. Bland-Altman plots for the method comparison for androstenedione (A,B), DHEA (C,D), and testosterone (E) with LC-MS/MS methods of commercial laboratories (B,D,E), and commercial immunoassays (A,C).

Dotted lines are 2 SD of the differences (concentrations in Figs. 2–4 expressed in μg/L).

stenedione was determined to be caused by the calibration (see online Supplemental Fig. 2). Comparison with direct immunoassay showed large discrepancies between the methods for DHEA and androstenedione (Fig. 2; see online Supplemental Fig. 2). Both immunoasays had proportional and constant biases, leading to overestimation of the concentration by the immunoassays, and were less sensitive compared to the LC-MS/MS method. Lower SE values were observed in comparisons with LC-MS/MS methods than with immunoassays. From the more than 50 steroids and steroid metabolites evaluated (see online Supplemental Table 1), trans-dehydrotestosterone produced a peak in the mass transition of DHEA (*m/z* 304–253) and testosterone (*m/z* 304–112); the peak was not present in the secondary mass transition of testosterone, and the ratio of the mass transitions of DHEA was outside of the expected range. The observed concentrations corresponded to 0.06% of the concentration of trans-dehydrotestosterone present in the samples. This interference can be detected through the evaluation of the ratios of concentrations determined from corresponding primary and secondary mass transitions *(22)*.

Evaluation of the storage stability of androgens showed acceptable stability in the samples stored at 4 °C, and −20 °C. In the samples stored at room temperature testosterone was stable, whereas the concentration of androstenedione decreased at a rate of approximately 15% per week and DHEA increased at a rate of approximately 5% per week. The increase of DHEA was likely related to partial hydrolysis of DHEA-sulfate, which is present in blood at significantly higher concentrations than DHEA. Recovery of the liquid/liquid extraction was 79%, 71%, and 80% for androstenedione, DHEA, and testosterone, respectively. Average absolute recovery, determined using 3 patient serum samples in the experiments with standard addition of the androgens, were 97%, 109%, and 97% for androstenedione, DHEA, and testosterone, respectively. No statistically significant difference in the recovery was observed between sets of paired (n = 7) serum and plasma samples (comparisons were performed with nonparametric Wilcoxon 2-group tests). No statistically significant differences in concentrations were observed in serum samples analyzed before storage and after storage for more than a year at −70 °C (nonparametric Wilcoxon 2-group tests). Evaluation of ion suppression showed negative peaks at the retention times of 1.25, 2.25, and 2.30 min; no ion suppression was observed in the retention window of the androgens.

The reference intervals that we determined with our method using samples from healthy children and adults grouped by TS and age are summarized in Tables 1 and 2, and plots of the median concentrations are

Table 1. Serum androgen reference intervals for males according to TS and age.

	N	Androstenedione, ng/L	DHEA, ng/L	Testosterone, ng/L
TS				
1	278	35–320	110–2370	16–150
2	131	79–480	370–3660	33–3030
3	140	140–870	750–5240	100–8510
4 and 5	204	270–1070	1216–67030	1620–8470
Age				
6–24 months	123	25–150 (<140)[a]	<2500 (<2170)	<370 (<280)
2–3 years	125	<110 (<100)	<630 (<430)	<150 (<130)
4–5 years	125	23–170 (<150)	<950 (<880)	<190 (<180)
6–7 years	125	10–290 (20–240)	60–1930 (80–1780)	<130 (10–130)
7–9 years	206	30–300	100–2080	17–81
10–11 years	140	70–390	320–3080	23–1650
12–13 years	143	100–640	570–4100	30–6190
14–15 years	141	180–940	930–6040	310–7330
16–17 years	136	300–1130	1170–6520	1580–8260
18–40 years	70	330–1340	1330–7780	2070–6970
40–67 years	61	230–890	630–4700	1320–6930

[a] Values in parentheses correspond to the central 90% of the distribution.

shown in Figs. 3 and 4. Concentrations of all 3 androgens (Tables 1 and 2) were found to depend on age, sex, and TS. In prepubertal children, concentrations of androstenedione and DHEA decreased between the ages of 6 months and 1 year, reached a nadir in 2- to 3-year-olds, and then started increasing and reached concentrations observed at the age of 12 months again by the age of 7 years (Fig. 4). Concentrations of testosterone in both sexes did not change substantially between the ages of 6 months and 7 years. Concentrations of androstenedione and DHEA started increasing in girls approximately 1 year earlier than in boys (Fig. 3). At the age of 6 years median concentrations of androstenedione and DHEA were twice as high in girls compared to boys.

In older girls, concentrations of all 3 androgens reached adult values at TS 3, whereas in boys concentrations reached adult values at TS 4–5. The greatest increase in the concentrations of the androgens was observed in girls during the transition to TS 2 and in boys during the transition to TS 3. In both sexes the median concentrations of androgens peaked at the age of 20–30 years and gradually declined with advancing age. Of the 3 androgens, the steepest age-dependent decline was observed for DHEA in both sexes (see online Supplemental Fig. 1).

In premenopausal women the median concentrations of testosterone were age independent, and remained within a range of 240–290 ng/L between TS 3 and menopause. In premenopausal and (postmenopausal) women the median concentrations of androstenedione, DHEA, and testosterone were 810 (360), 3000 (1670), and 270 (180) ng/L, respectively, and the median ratios of androstenedione/DHEA and testosterone/androstenedione were 0.28 (0.21) and 0.31 (0.47), respectively. In postmenopausal women testosterone concentrations were also age independent, whereas concentrations of androstenedione decreased at a rate of approximately 10% and DHEA decreased at a rate of approximately 25% per decade of life, and the median ratio of testosterone/androstenedione increased at a rate of 30% per decade of life.

In adult men, the median concentrations of androstenedione, DHEA, and testosterone were 440, 2000, and 3700 ng/L, respectively; median ratios of androstenedione/DHEA and testosterone/androstenedione were 0.21 and 7.18. Declines in all 3 androgens in men were more gradual compared to women. Median concentrations of testosterone in men declined starting from the age of 40 years at a rate of approximately 20% per decade of life. In men the median concentrations of androstenedione and DHEA reached maximum values at the age of 20 to 30 years and then declined at a rate of approximately 5% per decade of life for androstenedione and at a rate of approximately 10% for DHEA.

Table 2. Serum androgen reference intervals for females according to TS, menstrual status, and age.

	N	Androstenedione, ng/L	DHEA, ng/L	Testosterone, ng/L
TS				
1	296	45–510	140–2760	19–170
2	120	150–1370	830–4870	45–400
3	135	370–2240	1080–7560	100–630
4 and 5	205	350–2050	1240–7880	110–620
Menstrual status				
Before menarche	413	48–1080	160–4050	19–350
After menarche, ≤18 years	323	330–2130	1110–7700	100–630
Premenopausal, >18 years	104	260–2140	1120–7430	90–550
Postmenopausal	86	130–820	600–5730	47–320
Age				
6–24 months	92	<150 (<130)[a]	<1990 (<780)	<90 (<90)
2–3 years	126	<160 (<130)	<850 (<680)	<200 (<140)
4–5 years	127	20–210 (<180)	<1030 (70–770)	<300 (10–200)
6–7 years	131	20–280 (40–300)	<1790 (120–1520)	<70 (10–60)
7–9 years	206	40–420	140–2350	10–110
10–11 years	148	90–1230	430–3780	29–320
12–13 years	142	240–1730	890–6210	60–500
14–15 years	143	390–2000	1220–7010	60–520
16–17 years	138	350–2120	1420–9000	90–580
18–40 years	74	NA[b]	1330–7780	NA[b]
>40 years	116	NA[b]	630–4700	NA[b]

[a] Values in parentheses correspond to central 90% of the distribution.
[b] NA, not applicable because menstrual-status dependent.

In females, the testosterone/androstenedione ratio (representative of 17βHSD activity) did not change between TS 1 and 5, but increased with advanced age, especially after menopause. In males the testosterone/androstenedione ratio reached adult values by TS 3. After age 40 years, the ratio declined with advancing age. The ratio of androstenedione/DHEA (representative of 3βHSD activity) was not associated with age or sexual development in males (Fig. 3), whereas it had a tendency toward higher values in women with advancing age, especially after menopause.

Discussion

Analysis of oxime derivatives provided greater sensitivity compared to methods using protonated molecular ions of nonderivatized androgens. Derivatization enhanced the ionization efficiency for all targeted androgens and resulted in a 3- to 5-fold gain in the sensitivity. The sensitivity for DHEA was not enhanced as much as that for testosterone and androstenedione, because DHEA lacks high-intensity unique product ions in its mass spectrum. The only transition with adequate specificity for DHEA-oxime was *m/z* 304–253, and for this reason the secondary mass transition for DHEA was the same as the primary transition, but acquired at a lower collision energy *(22)*. Efficient ionization and fragmentation of androstenedione and testosterone are likely explained by the presence of conjugated double bonds, which cause formation of a resonance structure, leading to more efficient ionization *(17, 26)* and cleavage of the bonds at carbons 5 and 10 (*m/z* 124), and 4 and 10 (*m/z* 112).

Androgens analyzed by this method are structurally similar, with consequent similar chromatographic retention properties. These similarities lead to difficulties in analysis because DHEA and testosterone are isomers, sharing the same product ion (transition *m/z* 304–124); the second isotopic peak (A+2) of androstenedione monooxime is an isobar of testosterone and DHEA, which has the same product ions as testosterone and DHEA; and dihydrotestosterone is an isobar of the commercially available internal standard d_2-DHEA. The best selectivity

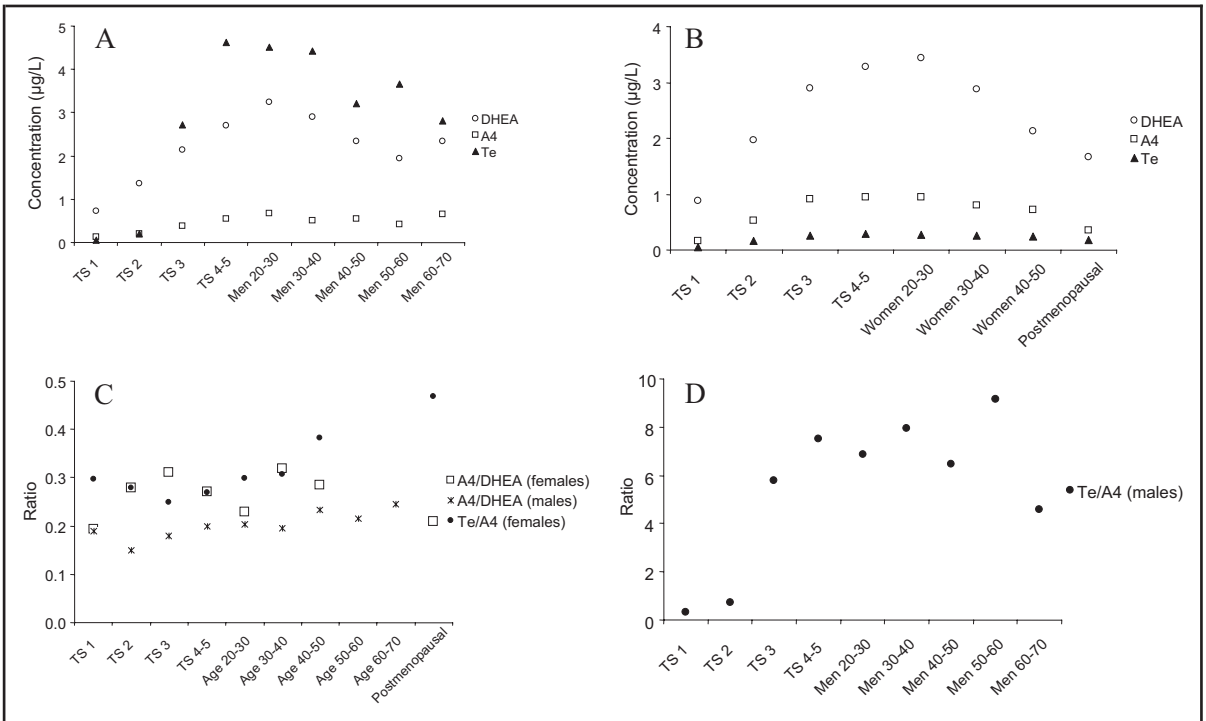

Fig. 3. Median concentrations of androstenedione (A4), DHEA and testosterone (Te) in samples from men (A), and women (B) according to TS or age in years, and ratios of concentrations of the androgens (C, D).

for chromatographic separation was achieved with 2-dimensional separation achieved by use of a phenyl-modified stationary phase as the first dimension and C18 as the second dimension. Compared to isocratic separation *(17)* performed by using a C18 column, the 2-dimensional separation resulted in a 4- to 5-fold improvement in the signal-to-noise ratio for testosterone.

On the basis of the high sensitivity of this method, its good agreement with other LC-MS/MS methods, and within-individual and between-individuals biologic variability of androgen concentrations *(26)*, this method is suitable for measurement of androgens in serum samples of both genders and in all age groups.

Reference intervals for androgens in children are important because concentrations of sex steroids vary with sex and age. There have been relatively few reports of androgen concentrations in healthy children *(27–32)* because commercial assays for measurement of androgens lack specificity and sensitivity with pediatric samples. We report data on concentrations of androstenedione, DHEA, and testosterone in more than 2500 healthy children from early childhood through puberty.

Concentrations of testosterone in 24 children from the 6-month through 6-year-old group were higher than expected. To exclude a deficiency of one of the enzymes in the pathway of steroid biosynthesis as a cause, the samples were tested for adrenal steroids *(27)*. Concentrations of adrenal steroids in these samples were within their TS-specific reference intervals. One explanation for increased testosterone concentrations could be stress response to the induction of anesthesia. To account for this effect we included the central 90% reference limits in addition to the robust reference intervals in Tables 1 and 2.

Given the similar distributions of DHEA concentrations in men and women (see online Supplemental Fig. 1), we established a single reference interval for both sexes. Between-sex differences in the distribution of androstenedione, as well as the differences in the trends for age-specific changes, warranted sex-specific reference intervals (Tables 1, 2). Compared to our earlier published data on LC-MS/MS reference intervals of testosterone *(17)*, in this study we used a considerably larger number of pediatric samples, as well as samples from children age 6 months through 6 years. Our reference intervals for testosterone in children agreed well with our previous data *(17)* and the reference intervals established by Soldin et al. *(32)*, whereas Soldin et al were not able to distinguish age and sex-specific differences in DHEA using their method. Likely explanations are different populations and smaller numbers of study participants *(32)*.

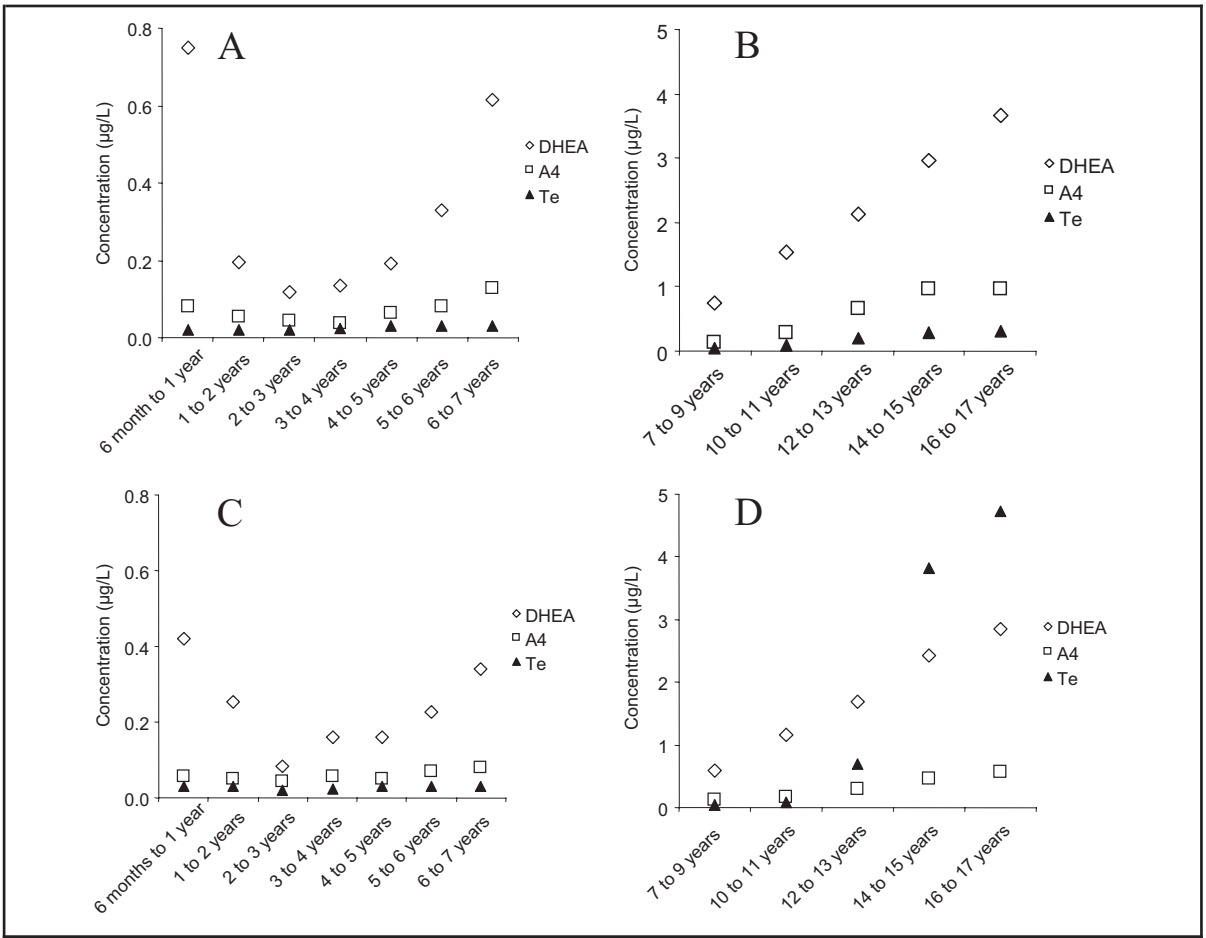

Fig. 4. Median concentrations of androstenedione (A4), DHEA and testosterone (Te) in samples from girls age 6 months through 6 years (A), girls age 7 through 17 years (B), boys age 6 months through 6 years (C), and boys age 7 through 17 years (D).

In summary, for LC-MS/MS quantification of androstenedione, DHEA, and testosterone in serum, we achieved the best sensitivity using derivatization with hydroxylamine in conjunction with a 2-dimensional chromatographic separation. Our reference intervals for androstenedione, DHEA, and testosterone for prepubertal children and postmenopausal women support the need for high-sensitivity measurements of androgens in these groups. To ensure harmonization between measurements of androgens among different laboratories it is important to have commercially available primary and matrix-based reference materials and reference measurement procedures *(33)*.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: W.L. Roberts, ARUP Laboratories.
Stock Ownership: None declared.
Honoraria: W.L. Roberts, Siemens Healthcare Diagnostics and Beckman Coulter.
Research Funding: A.L. Rockwood, ARUP Laboratories; W.L. Roberts, Siemens Healthcare Diagnostics and Beckman Coulter.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank ARUP Institute for Clinical and Experimental Pathology for supporting this project, A. Wilson for assistance with establishing reference intervals, and J. Simmons for assistance with determining TS of the children who participated in the study.

References

1. Achermann JC, Hughes IA. Disorders of sex development. In: Kronenberg H, Melmed S, Polonsky K, Larsen R. Williams textbook of endocrinology. 11th ed. Philadelphia (PA): Saunders, 2008; p 783–848.
2. Rosenfield RL. Pilosebaceous physiology in relation to hirsutism and acne. Clin Endocrinol Metab 1986;15:341–62.
3. Waggoner W, Boots LR, Azziz R. Total testosterone and DHEAS levels as predictors of androgen-secreting neoplasms: a populational study. Gynecol Endocrinol 1999;16:394–400.
4. Escobar-Morreale HF, San Millan JL, Smith RR, Sancho J, Witchel SF. The presence of the 21-hydroxylase deficiency carrier status in hirsute women: phenotype-genotype correlations. Fertil Steril 1999;72:629–38.
5. Speiser PW, White PC. Medical progress: congenital adrenal hyperplasia. N Engl J Med 2003;349: 776–88.
6. Iughetti L, Predieri B, Ferrari M, Gallo C, Livio L, Milioli S, et al. Diagnosis of central precocious puberty: endocrine assessment. J Pediatr Endocrinol Metab 2000;13:709–15.
7. Traggiai C, Stanhope R. Delayed puberty. Best Pract Res Clin Endocrinol Metab 2002;16:139–51.
8. Carel JC, Léger J. Precocious puberty. N Engl J Med 2008;358:2366–77.
9. Kulin HE, Finkelstein JW, D'Arcangelo MR, Susman EJ, Chinchilli V, Kunselman S, et al. Diversity of pubertal testosterone changes in boys: constitutional delay in growth and/or adolescence. J Pediatr Endocrinol Metab 1997;10:395–400.
10. Rossi C, Calton L, Hammond G, Brown HA, Wallace AM, Sacchetta P, Morris M. Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography-tandem mass spectrometry. Clin Chim Acta 2010;411:222–8.
11. Barrett-Connor E, Garland C, McPhillips JB, Khaw K-T, Wingard DL. A porspective, population-based study of androstenedione, estrogens and prostate cancer. Cancer Res 1990;50:169–173.
12. Duxbury K, Gallagher L, Keevil B. The impact of simultaneous measurement of testosterone and androstenedione in women with suspected androgen excess. Clin Chem 2007;53:804–5.
13. Herold DA, Fitzgerald RL. Immunoassays for testosterone in women: better than a guess? Clin Chem 2003;49:1250–1.
14. Lee JS, Ettinger B, Stanczyk FZ, Vittinghoff E, Hanes V, Cauley JA, et al. Comparison of methods to measure low serum estradiol levels in postmenopausal women. J Clin Endocrinol Metab 2006;91:3791–7.
15. Taieb J, Mathian B, Millot F, Patricot MC, Mathieu E, Queyre N, et al. Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography—mass spectrometry in sera from 116 men, women, and children. Clin Chem 2003;49:1381–95.
16. Wang C, Catlin D, Demers L, Starcevic B, Swerdloff RS. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography tandem mass spectrometry. J Clin Endocrinol Metab 2004;89:534–43.
17. Kushnir MM, Rockwood AL, Roberts WL, Pattison EG, Bunker AM, Fitzgerald RL, Meikle AW. Performance characteristics of a novel tandem mass spectrometry assay for analysis of testosterone in serum. Clin Chem 2006;52:120–8.
18. Guo T, Taylor RL, Singh RJ, Soldin SJ. Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin Chim Acta 2006;372:76–82.
19. Soldin SJ, Soldin OP. Steroid hormone analysis by tandem mass spectrometry. Clin Chem 2009;55: 1061–6.
20. Kushnir MM, Rockwood AL, Bergquist J. Liquid chromatography tandem mass spectrometry applications in endocrinology. Mass Spectrom Rev 2010;29:480–502.
21. Thienpont LM, Van Uytfanghe1 K, Stockl D, Blincko S, Ramsay CS, Xie H, et al. State-of-the-art of serum testosterone measurement by isotope dilution-liquid chromatography/tandem mass spectrometry. Clin Chem 2008;54:1290–7.
22. Kushnir MM, Rockwood AL, Nelson GJ, Yue B, Urry FM. Assessing analytical specificity in quantitative analysis using tandem mass spectrometry. Clin Biochem 2005;38:319–27.
23. Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49:1041–4.
24. Horn PS, Pesce AJ, Copeland BE. A robust approach to reference interval estimation and evaluation. Clin Chem 1998;44:622–31.
25. Cornbleet PJ, Gochman N. Incorrect least-squares regression coefficients in method-comparison analysis. Clin Chem 1979;25:432–8.
26. Westgard QC. Biological Variation Database specifications. http://www.westgard.com/biodatabase1.htm (Accessed May 2010).
27. Kushnir MM, Rockwood AL, Roberts WL, Owen WE, Bunker AM, Meikle AW. Development and performance evaluation of a tandem mass spectrometry assay for four adrenal steroids. Clin Chem 2006;52:1559–67.
28. Ankarberg C, Norjavaara E. Diurnal rhythm of testosterone secretion before and throughout puberty in healthy girls: correlation with 17beta-estradiol and dehydroepiandrosterone sulfate. J Clin Endocrinol Metab 1999;84:975–84.
29. Ankarberg-Lindgren C, Norjavaara E. Changes of diurnal rhythm and levels of total and free testosterone secretion from pre to late puberty in boys: testis size of 3 ml is a transition stage to puberty. Eur J Endocrinol 2004;151:747–57.
30. Meikle AW, Kushnir MM, Rockwood AL, Pattison EG, Terry AH, Sandrock T, et al. Adrenal steroid concentrations in children seven to seventeen years of age J Pediatric Endocrinol Metab 2007; 20:1281–91.
31. Elmlinger MW, Kuhnel W, Wormstall H, Döller PC. Reference intervals for testosterone, androstenedione and SHBG levels in healthy females and males from birth until old age. Clin Lab 2005;51:625–32.
32. Soldin OP, Sharma H, Husted L, Soldin SJ. Pediatric reference intervals for aldosterone, 17alpha-hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D3 using tandem mass spectrometry. Clin Biochem 2009; 42:823–7.
33. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Utility, limitations, and pitfalls in measuring testosterone: an endocrine society position statement. J Clin Endocrinol Metab 2007;92:405–13.