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Pediatric reference intervals for aldosterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, testosterone and 25-hydroxy vitamin D₃ using tandem mass spectrometry

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Abstract

Objectives—To determine age and sex-specific pediatric reference intervals for aldosterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, testosterone, and 25-hydroxy vitamin D₃.

Background and design—Reference intervals were determined for neonates and children 0–18 years of age. The study was conducted at both Children's National Medical Center and Georgetown University using outpatient blood samples obtained between January 1, 2004 and June 30, 2008.

Methods—Serum samples were analyzed using isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) with deuterium-labeled internal standards at Children's National Medical Center and Georgetown University Medical Center Bioanalytical Core Laboratory.

Results and conclusions—This is the first study to provide pediatric reference intervals of steroid hormones for children from birth to 18 years of age using LC/MS/MS. Reference intervals were established for aldosterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, testosterone, and 25-hydroxy vitamin D₃. All the analytes exhibited at least some age dependence. Sex differences between early and late childhood and adolescence were found for 17 α -hydroxyprogesterone and testosterone. Seasonal differences were apparent for 25-hydroxy vitamin D₃.

Keywords

Reference values; Sex differences; Children; DHEA; Androgens; Steroids; Hormones; Tandem mass spectrometry

Introduction

Pediatric reference intervals are crucial for patient care in distinguishing between healthy and diseased patients, especially for analytes whose concentrations vary with sex and age. Isotope dilution tandem mass spectrometry is the most advanced method allowing the simultaneous detection and quantitation of steroid profiles [1,2]. Tandem mass spectrometry has several advantages over immunoassays for the measurement of steroids which include better specificity and ability to quantify numerous steroids in a single run [1,2].

Aldosterone, synthesized in the adrenal cortex, is a prominent mineralocorticoid that affects the kidneys by increasing the permeability of the apical (luminal) membrane to potassium and sodium and activating the basolateral Na⁺/K⁺ pumps [3,4]. Aldosterone is associated with vascular-related pathologies such as hypertension [5]. Addison's disease is a rare disorder in which the adrenal gland produces insufficient amounts of glucocorticoids and often mineralocorticoids. Hyperaldosteronism is far more common (Conn's Syndrome) and causes hypertension as a result of sodium retention. Hypokalemia may also result [6].

17 α -hydroxyprogesterone is a steroid hormone synthesized predominately in the adrenal glands. Mutations in the genes coding for the enzymes 21-hydroxylase and 11 β -hydroxylase can result in exceptionally high levels of 17 α -hydroxyprogesterone which together with elevations in androstenedione and testosterone are the hallmarks of congenital adrenal hyperplasia (CAH) in the newborn [7–9]. 21-hydroxylase deficiency accounts for approximately 95% of cases of CAH [10]. Deficiencies of 21-hydroxylase, though rare, can be detected through neonatal screenings and treated [8].

Dehydroepiandrosterone (DHEA) is secreted from the zona reticularis of the adrenal glands [11] and is a precursor for both androgen and estrogen synthesis. The specific physiological function of DHEA is unclear, but it is believed to have an androgenic or estrogenic effect [12]. DHEA insufficiency may indicate Addison's disease or hypopituitarism [13]. DHEA levels sharply decrease in adulthood and low concentrations have been associated with diabetes mellitus and cardiovascular diseases [14].

Testosterone, the principal male sex hormone and an anabolic steroid responsible for metabolic function in multiple body systems, is an androgen secreted by the testes, ovaries and adrenal glands [15,16]. Elevated serum testosterone concentrations indicate overproduction by the testes or by the adrenals and can result in premature pubarche [17,18]. Mildly elevated concentrations of DHEA, DHEAS, androstenedione and testosterone are found in children with premature adrenarche [18]. High testosterone levels in neonates may correlate with later autism spectrum conditions [19].

25-hydroxy vitamin D₃ is a steroid hormone synthesized by the skin following exposure to UV rays, and is associated with calcium regulation and bone growth [20]. Severe 25-hydroxy vitamin D₃ deficiency can lead to rickets, osteoporosis and cancer [21]. 25-hydroxy vitamin D₃ plays a significant role in preventing tumor formation and growth as well [21]. Recent studies have highlighted the clinical importance of 25-hydroxy vitamin D₃ by revealing deficiencies in people with dark skins and correlating diseases such as cancer with inadequate 25-hydroxy vitamin D₃ concentrations.

Materials and methods

Blood specimens on which one or more steroids had been ordered were obtained from Children's National Medical Center's outpatient population from January 2004 to June 2008. All identifiers except age and sex were removed and ordered steroids were quantified on plasma or serum samples. Institutional review board approval was obtained.

LC/MS/MS Analysis: All steroid hormones were analyzed by methods developed in our laboratories and published previously [1,2]. Briefly, deuterium-labeled internal standards were used. These included: aldosterone-d6 obtained from IsoSciences (King of Prussia, PA); 4-pregnen-17 α -ol-3,20-dione-2, 2,4,6,6,21,21,21-d8 (17 α -hydroxyprogesterone-d8), dehydroepiandrosterone- 16,16-d2 (DHEA-d2) obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), testosterone-1, 2-d2 (testosterone-d2) obtained from Cambridge Isotope Laboratory, Inc. (Andover, MA) and 25-hydroxyvitamin D₃-d6 was from AS Vitas (Oslo, Norway). Acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). HPLC-grade methanol and Optima-grade toluene were from Fisher Scientific (Fair Lawn, NJ). Deionized water was prepared from Barnstead BPURE Water System (Dubuque, IA).

Two hundred microliters of serum was placed into a 1.5-mL conical plastic tube. Ten microliters of acetonitrile containing 60 ng cortisol was added. This was done to saturate cortisol binding globulin which would otherwise avidly bind aldosterone. Three hundred microliters of acetonitrile containing the internal standards was added to the serum sample. The samples were then vortexed for 30 s and centrifuged at 13,000 rpm for 10 min. 450 microliters of the supernatant was placed into autosampler vials and diluted with 900 μ L of water. This vial was well mixed, and 1 mL was injected onto the C-8 Supelco column. Sample preparation was conducted at room temperature and analysis was performed using the API-5000 triple-quadrupole mass spectrometer using an atmospheric pressure photoionization source 3 Shimadzu LC-10ADvp pumps, a Shimadzu SIL-HT autosampler, and a Shimadzu DUG-14A degasser. The API-5000 was operated in multiple reaction monitoring (MRM) positive ion mode to determine all steroid concentrations except for aldosterone which was quantified in the negative ion mode. Nitrogen served as the curtain nebulizer, collision, and lamp gases.

Statistical analysis

Reference intervals were established for all analytes on a cohort of healthy outpatient pediatric population using the Hoffmann approach (Fig. 1) [22]. As the Hoffmann approach automatically eliminates the diseased population, sick out-patients would automatically be excluded from the calculation of each reference interval. Results obtained by the Hoffmann approach were almost identical to those obtained using the percentile approach as the population studied were essentially healthy outpatients.

Results

Due to small sample size limitations the data for aldosterone could only be divided into two groups, namely males and females from 0–8 years, and males and females greater than 8 years of age (Table 1). The 0–8 years age reference interval ranged between 1 and 196 pg/mL while the reference interval for the group of children 8 years and older had a range from 2 to 200 pg/mL.

Six specific reference intervals were established for 17 α -hydroxyprogesterone (Table 2). Males and females were combined in the age group 0 to 6 months. The 0 to 6 month interval had the widest reference interval (25–248 ng/dL). The lower limit for this reference interval was higher than the 2.5th percentile for any other reference interval for children older than 6 months. The 97.5th percentile for the 0 to 6 month range was the highest and decreased with age for males and for females. For females 10 to 18 years of age the reference intervals (15–137 ng/dL) were higher than for females younger than 10 years of age and for males of the same age range. Again, due to sample size limitations, only one reference interval was established for DHEA. The 2.5th percentile value for DHEA was 10 ng/dL and the 97.5th percentile value was 565 ng/dL (Table 3).

For testosterone, 17–19 year old males had the highest 2.5th percentile value. 15–16 year old males had the highest 97.5th percentile value. In females, the greatest increase in the 2.5th percentile value occurred between the 6–9 year age group and the 10–14 year age group. The greatest increase in the 97.5th percentile value also occurred between the 6–9 year age group and the 10–14 year age group. In males, the greatest increase in 2.5th percentile value occurred between the 13–14 year age group and the 15–16 year age group. The greatest increase in the 97.5th percentile value occurred between the 7–9 year age group and the 10–12 year age group.

Separate reference intervals for 25-hydroxy vitamin D₃ were determined based on age and season. The highest 2.5th percentile value occurred at 6 months to 1 year (Table 5a). The lowest 2.5th percentile value occurred at 0 to 3 months. The highest 97.5th percentile value occurred at 3 to 6 months. The lowest 97.5th percentile value occurred in the 13 to 15 year age group.

For each of the seasonal reference intervals, summer 2.5th and 97.5th percentile values were greater than winter 2.5th and 97.5th percentile values (Table 5b).

Discussion

This manuscript is the first to obtain pediatric reference intervals for aldosterone, 17 α -hydroxyprogesterone, DHEA, testosterone and 25-hydroxy vitamin D₃ by a specific isotope dilution tandem mass spectrometric method. The lack of specificity of immunoassays for drug and steroid measurement has been well documented [1,2,23–36], and therefore these new steroid reference intervals using the more specific tandem mass spectrometry methodology will provide more accurate results. Due to the low number of subjects in the groups for DHEAS, androstenedione and progesterone reference intervals could not be calculated for these analytes.

Calculated reference intervals for aldosterone are shown in Table 1. Prior studies have shown that aldosterone decreases significantly in the first year of life [37]. The reference interval for 0–7 year old males and females ranged from 1 to 197 pg/mL, while the range in the older children (8–18 years) is 2–200 pg/mL (Table 1). These intervals are similar to the values found in adults [38].

However, pediatric reference intervals for many analytes can vary significantly by both sex and age from those found in adults, and are of great importance in differentiating between the “healthy” and the “diseased” populations [37–45].

17 α -hydroxyprogesterone concentrations remained fairly constant in infants 6 months of age and older. In females from 6 to 10 years of age 17 α -hydroxyprogesterone concentrations ranged from 6 to 62 ng/dL. The 97.5th percentile for this population was lower than that of females in the younger or older female groups (Table 2). This was similar to the intervals found in other studies using immunoassays [39].

Previous studies have demonstrated that DHEA concentrations are highest in neonates less than one month old. From birth, DHEA concentrations are known to decline until approximately 8 years of age [40]. DHEA is widely considered to be an indicator of aging [13]. In both males and females, no seasonal or diurnal trends have been found [41,42]. The 2.5th percentile was 10 ng/dL (Table 3) while the 97.5th percentile reached 565 ng/dL. The small number of subjects in the DHEA arm is suboptimal and necessitated the use of a singular age range of 0–18 years. Despite this problem, the reference intervals are in agreement with the published literature [43].

Testosterone concentrations for pre-pubertal females were similar to values found in the published literature (Table 4). As expected, testosterone concentrations are higher in males than females for all age groups. Testosterone concentrations increase sharply at the onset of puberty in males (20 fold) and females (2 fold). The 97.5th percentile for 10–14 year old females and 10–12 year old males increased to 50 ng/dL and 418 ng/dL respectively. The intervals measured by tandem mass spectrometry are lower than those established by immunoassays [44]. Diurnal variations for aldosterone [45,46] and testosterone [45] have been observed.

Two separate sets of reference intervals were calculated for 25-hydroxy vitamin D₃. In the first group (Table 5a) the seasonal differences were not taken into consideration and the resulting reference intervals established were lower than the clinically recommended concentrations (30–100 ng/mL). Reference intervals were lower for the first few years of life, likely because of social behavior in North American society where infants and toddlers are protected from sunlight. This observation is supported by the literature [47].

A separate set of reference intervals were determined for 25-hydroxy vitamin D₃ based on both age and season (summer months being June–August and December–February winter months) [48,49]. The seasonal summer and winter analysis yielded consistent and interesting results illustrated in Table 5b. All four ranges showed higher reference intervals during the summer months when compared to the winter months. These results have been noted in previous studies [50]. However, it is noted that the reference intervals had 2.5th–97.5th percentiles range between 7 and 57 ng/mL, well below the currently recommended ranges of 30–100 ng/mL [47–49].

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**% Cumulative Frequency vs. Log of 25-hydroxy Vitamin D₃ Results
in 3-10 year old Males and Females**

Fig. 1.
Hoffmann plot of 25-hydroxy vitamin D₃.

Table 1

Reference Intervals for aldosterone (pg/mL)

Males and females			
Age	<i>n</i>	2.5%	97.5%
0–8 years	121	1	197
>8 years	172	2	200

Table 2Reference Intervals for 17 α -hydroxyprogesterone (ng/dL)

Age	<i>n</i>	2.5%	97.5%
<i>Males</i>			
0 to <6 months	119	25	248
6 months to <18 years	153	7	100
<i>Females</i>			
0 to <6 months	119	25	248
6 months to <6 years	132	3	107
6 to <10 years	182	6	62
10 to <18 years	144	15	137

Table 3

Reference Intervals for DHEA (ng/dL)

Males and females			
Age	<i>n</i>	2.5%	97.5%
0 to <18 years	122	10	565

Table 4

Reference intervals for testosterone (ng/dL)

Age	<i>n</i>	2.5%	97.5%
<i>Females</i>			
0–5 years	127	2	10
6–9 years	171	5	13
10–14 years	190	14	50
15–16 years	175	12	53
17–18 years	123	16	50
<i>Males</i>			
0–6 years	158	4	31
7–9 years	148	4	25
10–12 years	188	5	418
13–14 years	245	6	647
15–16 years	209	42	880
17–19 years	123	121	823

Table 5aReference intervals for 25-hydroxy vitamin D₃ (ng/mL) *

Males and females			
Age	n	2.5%	97.5%
0 to <3 months	131	5	42
3 to <6 months	135	9	60
6 months to <1 year	147	18	58
1 to <3 years	394	15	54
3 to <10 years	619	14	46
10 to <13 years	286	11	50
13 to <15 years	275	10	44
15 to <18 years	390	8	45
>18 years	421	8	56

*
All seasons.

Table 5bSummer vs. winter reference intervals for 25-hydroxy vitamin D₃ (ng/mL)

Males and females			
Age	n	2.5%	97.5%
Summer 0 to <3 years	227	15	57
Winter 0 to <3 years	202	10	57
Summer 3 to <10 years	195	17	52
Winter 3 to <10 years	137	11	38
Summer 10 to <18 years	293	12	49
Winter 10 to <18 years	260	8	39
Summer >18 years	134	12	50
Winter >18 years	122	7	44