

## Measurement of androstenedione levels by an in-house radioimmunoassay

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### Abstract

An in-house radioimmunoassay (RIA) for the measurement of androstenedione levels in serum was established and validated. Levels of androstenedione were measured by RIA using serum samples from various normal population groups and patients with congenital adrenal hyperplasia (CAH). Analytical recovery and linearity results were >95%, while intra- and inter-assay CVs were <10% and <22% respectively. The assay sensitivity was 0.5 nmol/l or 25 fmol/tube. In normal population groups, the highest androstenedione levels were found in preterm neonates (1.6-12.4 nmol/l), followed by adult females (1.5-10.2 nmol/l), adult males (1.6-8.0 nmol/l) and term neonates (0.8-8.8 nmol/l), while the lowest values were observed in prepubertal children (0.5-3.4 nmol/l). There were no significant differences in diurnal variation and between follicular and luteal phases. The range of androstenedione levels in untreated or poorly controlled CAH patients (7.6-355.0 nmol/l, median 42.5 nmol/l, n=20) were significantly higher (p<0.001) than the upper normal limit of 3.4 nmol/L for prepubertal children. The normal androstenedione reference ranges for paediatric and adult groups have thus been established.

**Keywords:** Androstenedione, radioimmunoassay, congenital adrenal hyperplasia (CAH).

### INTRODUCTION

The adrenal cortex which produces the precursors dehydroepiandrosterone (DHEA) and androstenedione, is the main source of androgens in children and in adult females. In adult males, however, the contribution of adrenal androgens is minimal compared to the large amounts synthesised and secreted by the testes. There is an increased production of adrenal androgens in patients with congenital adrenal hyperplasia (CAH), which is due to an enzymatic defect in steroidogenesis, causing accumulation of steroid precursor 17-alpha-hydroxyprogesterone (17-OHP) but lack of cortisol and/or aldosterone.<sup>2,3</sup> Indeed, masculinisation, virilisation and accelerated somatic and skeletal growth are the common clinical manifestations in CAH patients apart from gluco- and mineralo-corticoid deficiencies. Although the measurement of 17-OHP in serum or blood spots is considered as the best parameter for diagnosis of CAH patients,<sup>4,6</sup> it has been reported that androstenedione levels are better for monitoring the efficacy of replacement glucocorticoid therapy.<sup>7,8</sup> Unlike 17-OHP levels, single measurements of androstenedione are less affected by wide circadian variations and individual replacement doses and are thus more useful indices for

assessment of adequacy of adrenal suppression and therapeutic control.<sup>7,9</sup> In this present study, we established an in-house radio-immunoassay (RIA) for measurement of androstenedione and analysed serum samples in normal neonate, children and adult population groups and in CAH patients.

### MATERIALS AND METHODS

#### CHEMICALS

Steroids, activated charcoal Norit A, 2,5-diphenyl-oxazole (PPO), 1,4-bis(5-phenyl-2-oxazolyl) benzene (POPOP), Triton X-100 and buffer chemicals were purchased from Sigma Chemical Co., St. Louis, MO 63178 USA; Dextran T-70 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Extrelut from E. Merck, FRG and [1,2,6,7-<sup>3</sup>H] androst-4-ene-3,17-dione (specific activity 91.8 Ci/mmol) from Amersham International Plc, Bucks., UK. Toluene, ethanol, diethyl ether were obtained from May and Baker Ltd., UK., BDH Chemicals Ltd, UK., and E. Merck, FRG.

#### REAGENTS

*Assay buffer:* 0.05 Phosphate buffer pH 7.4, containing 14.5g disodium hydrogen

orthophosphate, 1.5g sodium dihydrogen orthophosphate, 0.1g thiomersal and 1g gelatin per litre.

**Dextran-coated charcoal (DCC):** Norit A activated charcoal (0.4%) 4.0 g and 0.4 g Dextran T-70 in 1 litre of assay buffer.

**Scintillant:** PPO 5.5 g, POPOP 0.1 g, 333 ml Triton X-100 mixed well and made up to final volume of 1 litre with toluene. Androstenedione standards: Standard solutions, 0.78 to 50 nmol/l, were prepared in assay buffer from a 1.0 mmol/l (286 ug/ml) stock solution of androstenedione in absolute ethanol.

**Antiserum:** Rabbit anti-androstenedione serum was donated by Dr SS Lynch from the Birmingham and Midland Hospital for Women, UK. The antiserum with an affinity constant of  $7.0 \times 10^9$  l/mol had the following cross-reactivities: androstenedione 6 beta-01 3.0%, 11 beta-01 2.0%, testosterone, DHEA, 11-deoxycortisol, 17-OHP, estrone, estradiol, estriol, pregnanediol, pregnanetriol and progesterone <0.1%, DHEAS, cortisol, cholesterol and androsterone <0.01%. This antiserum was used at a final working dilution of 1:15,000 in the RIA.

**Quality control sera:** Commercial QC serum samples from Bio-Rad, USA were used.

### SAMPLES

Blood samples were collected between 0800-1200h from normal subjects representing various neonatal, paediatric and adult population groups and from prepubertal CAH patients (n=20, males=7, females=13) attending the Endocrine Clinic, UKM. Neonatal samples were taken from term (n=27) and preterm (n=29) babies that were older than four days, while prepubertal Tanner 1 children (n=21) were 3-13 years (mean=8.3, SD=2.3) and pubertal Tanner 2-4 male subjects (n=27) were 12-16 years (mean=13.3, SD=1.4). Blood samples from adult males were also collected in the afternoon between 1500-1600h. The normal adults (males=69, females=64) were 20-48 years (mean=27.6, SD=7.3) and were staff and students of the Faculty of Medicine, UKM. Blood samples were allowed to clot and the sera obtained were stored at -20°C.

### RADIOIMMUNOASSAY

Extrelut columns containing 100 mg Extrelut were prepared using 1 ml disposable pipette tips.

Serum samples or standard solutions (150 ul) were pipetted onto the top of the columns and left for 20 min before extraction with 3 ml diethyl ether. Solvent extracts were evaporated by heating and air-blowing in a fume cupboard and the residue were reconstituted with 600 ul assay buffer in a 60°C water bath for 20 min. Duplicate 200 ul of the extract was put into plastic LP3 tubes which contained 100 uL [<sup>3</sup>H]androstenedione tracer (5-6 000 cpm) and 100 uL of antiserum diluted 3 750-fold in assay buffer. The mixture was left to react for 1 h at room temperature (26-28°C) and then incubated for 10 min in ice, after which 500 ul DCC suspension was added to all tubes except to the total count tubes. The tubes were centrifuged for 10 min at 3 000 rpm at 4°C and the supernatants were immediately decanted into scintillation vials. After adding 4 ml scintillant and mixing for 30 min, the vials were placed in a liquid scintillation analyser (Canberra-Packard TriCarb 1900CA, USA.) and radioactivity in each vial was counted for 5 min. Data collected onto computer diskettes was loaded into the LKB Wallac RIACalc program (Wallac Oy, Turku, Finland) for analysis.

### DATA ANALYSIS

Statistical analyses were performed with an IBM compatible computer utilising the Statistical Analysis Systems (SAS) software from SAS Institute Inc., USA. Normality of data distribution was examined by the Wilks-Shapiro test and Student's unpaired t test was used to compare group means, with reference ranges reported as mean  $\pm$  2SD. Correlation analyses were performed using linear regression. Values of p<0.05 were considered significant.

## RESULTS

### Performance of androstenedione RIA

**Analytical recovery and linearity:** Recoveries from analysis of serum samples with added androstenedione were (mean  $\pm$  SD) 98.2  $\pm$  3.0% and 95.0  $\pm$  7.3% (n=10) at 40.0 and 10.0 nmol/l respectively. Linearity analysis on serum samples diluted 1/2, 1/4, 1/8 and 1/16 gave recoveries of 99.4  $\pm$  7.0, 106.0  $\pm$  10.0, 91.5  $\pm$  13.6 and 91.5  $\pm$  8.7% (n=8) respectively.

**Reproducibility:** As shown in Table 1, intra-assay coefficient of variations (CVs) at mean concentrations of 2.8, 7.1 and 23.9 nmol/l were 9.6, 9.4 and 7.0% (n=10) respectively. Inter-assay CVs for the same pools ranged from 10.0

**TABLE 1: Reproducibility of androstenedione RIA**

Androstenedione (nmol/l)	Precision			
	Intra-assay		Inter-assay	
	n	CV (%)	n	CV (%)
2.8	10	9.6	22	21.5
7.1	10	9.4	22	16.9
23.9	8	7.1	12	10.0

**TABLE 2: Diurnal variation of androstenedione levels**

Time(h)	Androstenedione (nmol/l)			
	n	Mean	SD	Range
Morning 0900-1000	23	5.3	1.5	2.4 - 8.2
Afternoon 1500-1600	25	5.0	1.7	1.6-8.5

p>0.5 vs morning

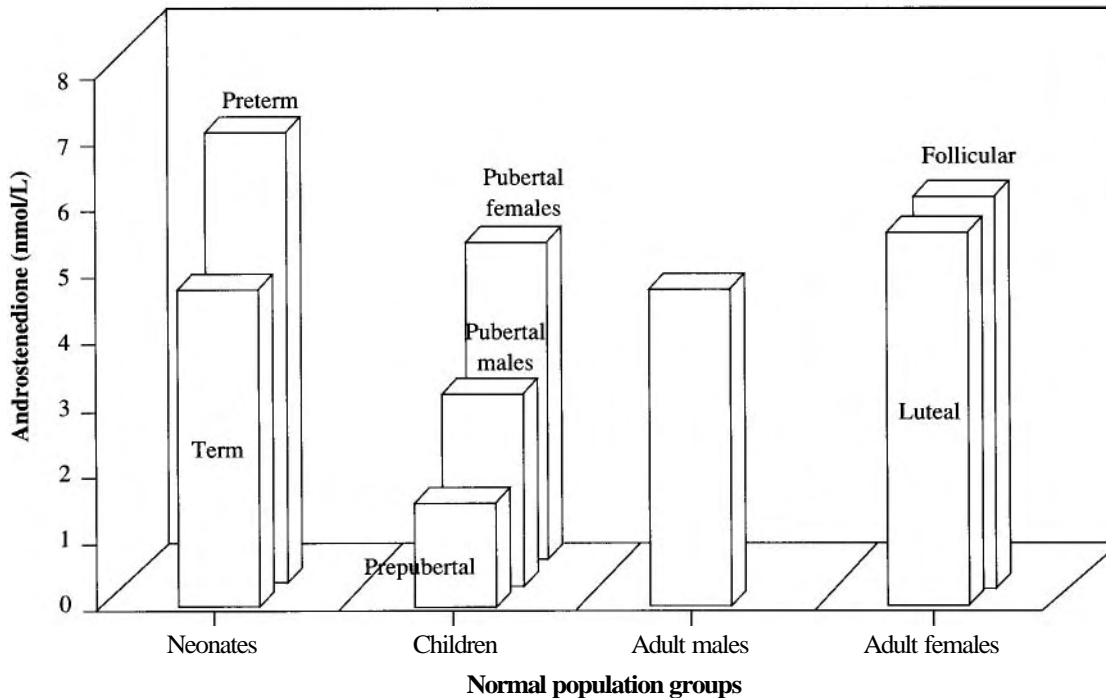
to 21.5% (n=22). The RIACalc intra-assay precision profiles (n=49) showed that the CVs were <10% over the working range of 0.78 - 50 nmol/l.

*Sensitivity:* The mean minimum detectable concentration (MDC) defined as the dose at 3SDs from zero binding was  $0.5 \pm 0.2$  nmol/l or  $25 \pm 10$  fmol/tube (n=23).

*Diurnal variation:* Serum samples that were collected in the morning (0900-1000h) had androstenedione levels,  $5.3 \pm 1.5$  nmol/l n=23 (Table 2) that were not significantly different from the range of hormone levels  $5.0 \pm 1.7$

nmol/l in blood samples collected in the afternoon (1500-1600h).

*Androstenedione levels in normal population groups and CAH patients:* The normal physiological levels of serum androstenedione in various population groups measured by the in-house RIA are shown in Figure 1 and summarised in Table 3. The highest androstenedione levels were found in preterm neonates (1.6-12.4 nmol/l), followed by adult females (1.5-10.2 nmol/l), adult males (1.6-8.0 nmol/l) and term neonates (0.8-8.8 nmol/l), while the lowest values were observed in prepubertal children (0.5-3.4 nmol/l). In females, there was no significant



**FIG. 1:** Normal physiological serum androstenedione levels of various population groups.

**TABLE 3: Serum androstenedione levels in normal population groups and CAH patients**

Subject group	Androstenedione (nmol/l)			
	n	Mean	SD	Range
Neonates:				
Preterm	24	6.8	2.7	1.6 - 12.4
Term	19	4.8 <sup>a</sup>	2.0	0.9 - 8.8
Children:				
Prepubertal	21	1.6 <sup>b</sup>	0.9	0.5 - 3.4
Pubertal males	27	2.9 <sup>c</sup>	1.7	0.5 - 6.2
Pubertal females	9	4.8 <sup>cd</sup>	2.3	0.5 - 9.3
Adults:				
Males	38	4.8 <sup>e</sup>	1.6	1.6 - 8.0
Females:-				
Follicular	22	5.9 <sup>f</sup>	2.2	1.5 - 10.2
Luteal	30	5.7 <sup>f</sup>	1.4	2.9 - 11.1
CAH patients	20	42.5(median) <sup>e</sup>		7.6 - 355.0

<sup>a</sup>p<0.01 vs preterm neonates

<sup>b</sup>p<0.001 vs term neonates

<sup>c</sup>p<0.01 vs prepubertal

<sup>d</sup>p<0.05 vs pubertal males

<sup>e</sup>p<0.001 vs pubertal males

<sup>f</sup>p<0.01 vs adult males

difference between follicular ( $5.9 \pm 2.2$  nmol/l) and luteal ( $5.7 \pm 1.4$  nmol/l) phases. The range of androstenedione levels in untreated or poorly controlled CAH patients (7.6-355.0 nmol/l, median 42.5 nmol/l, n=20) were significantly higher (p<0.001) than the upper normal limit of 3.4 nmol/l for prepubertal children.

Serum androstenedione levels were correlated

to 17-OHP values only in prepubertal children and in female follicular phase (Fig. 2 and Table 4). As shown in Table 4, a highly significant (p<0.001) correlation was obtained for serum samples from CAH patients ( $r=0.54$ ,  $y=0.13x + 7.0$ , n=56). The corresponding regression values for female follicular phase and prepubertal children were  $r=0.64$ ,  $p<0.01$  and  $r=0.59$ ,  $p<0.01$

**TABLE 4: Correlation of serum androstenedione and 17-OHP levels in normal population groups and CAH patients**

Subjects	Regression analysis			
	n	r	y = mx + c	P
Neonates:				
Preterm	19	0.22	0.04x + 6.7	>0.1
Term	19	0.09	0.04x + 4.8	>0.5
Children:				
Prepubertal	20	0.59	0.4x + 0.9	<0.01
Pubertal males	26	0.24	0.2x + 2.0	>0.1
Adults:				
Males	47	0.29	0.3x + 3.6	<0.05
Females:-				
Follicular	18	0.64	0.75x + 3.3	<0.01
Luteal	22	0.09	0.07x + 5.5	>0.5
Patients:				
CAH	56	0.54	0.13x + 7.0	<0.001

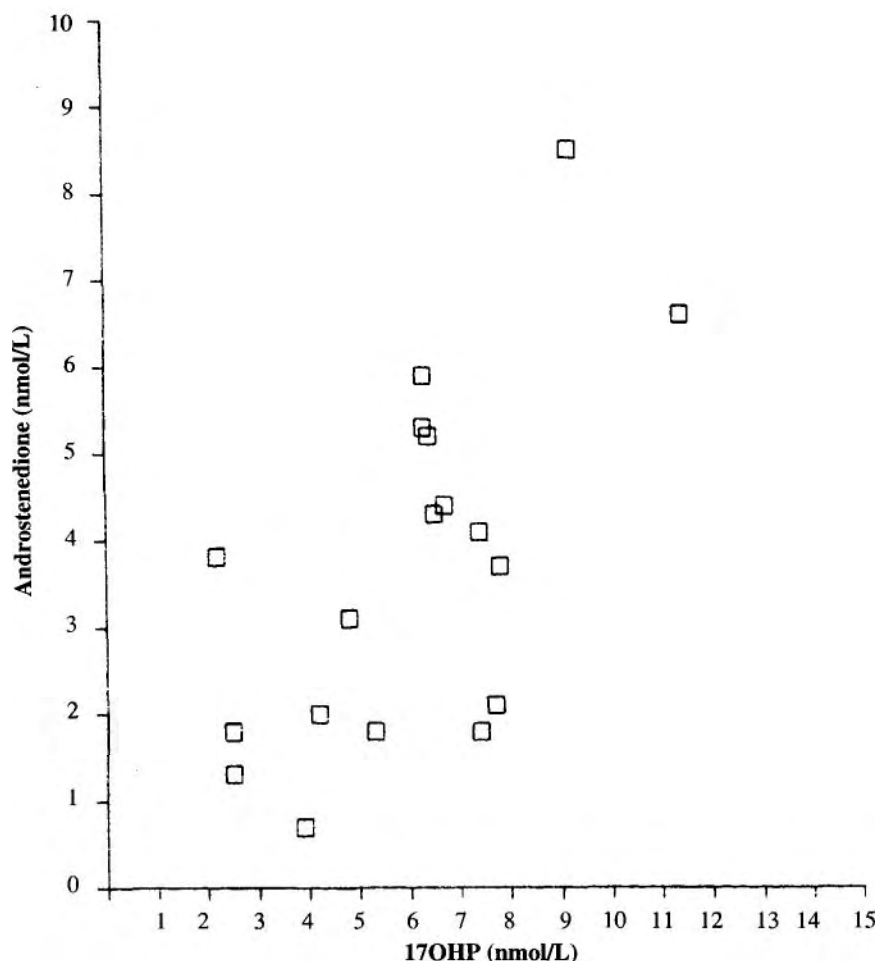


FIG. 2: Correlation of serum androstenedione and 17-OHP levels in adult females at follicular phase.

respectively. There was no correlation of androstenedione to 17-OHP levels in neonates, pubertal males and female luteal phase, while a weak correlation was observed for adult males ( $r=0.25$ ,  $p<0.05$ ).

## DISCUSSION

An in-house RIA for androstenedione was established and validated by recovery, linearity and precision studies which gave acceptable results; the sensitivity of the assay was  $0.5 \text{ nmol/l}$  or  $25 \text{ fmol/tube}$ . There was no diurnal variation observed in androstenedione levels in serum of adult males collected in the morning and afternoon. This result confirms that androstenedione levels, unlike 17-OHP, are less affected by circadian rhythms as reported earlier.<sup>6,9</sup> Similarly, there was less variation of androstenedione levels in the various normal population groups, in contrast to the marked distribution of serum 17-OHP concentrations.<sup>6,9</sup>

In adult females, androstenedione levels during follicular and luteal phases of the menstrual cycle were not significantly different from each other. This confirms that in normal physiology, the ovaries do not secrete androgen precursors. In contrast to testosterone, both adult and pubertal females have significantly higher androstenedione levels ( $p<0.01$ ,  $p<0.05$ ) than males. In males, adults have significantly higher androstenedione levels  $4.8 \pm 1.6 \text{ nmol/l}$ ,  $p<0.001$ , compared to those at puberty  $2.9 \pm 1.7 \text{ nmol/l}$ . However, the increase was only two fold, in contrast to the marked differences in testosterone distribution. Our androstenedione results from the normal paediatric population groups are also in agreement with earlier reports by Wallace *et al*<sup>12</sup> and Thomson *et al*<sup>11</sup>; preterm neonates had the highest levels followed by term neonates and prepubertal children. Our normal reference range for prepubertal children  $0.5\text{-}3.4 \text{ nmol/l}$  is comparable to the range of  $0.14\text{-}2.40 \text{ nmol/l}$  reported by Otten *et al*.<sup>8</sup>

The correlation ( $r=0.84$ ,  $p<0.001$ ) of serum androstenedione with 17-OHP levels in normal prepubertal children had been previously reported by Hughes and Winter.<sup>13</sup> In this study, almost similar correlations were observed in this group of children ( $r=0.59$ ,  $p<0.01$ ) and also in adult females during follicular phase ( $r=0.64$ ,  $p<0.01$ ). Androstenedione and 17-OHP levels did not correlate with each other in adult females at luteal phase and in pubertal males because of increased gonadal contribution of 17-OHP or androstenedione to existing circulating levels from the adrenal glands. In CAH patients, a highly significant ( $p<0.001$ ) correlation between androstenedione and 17-OHP levels ( $r=0.54$ ,  $y=0.13x + 7.0$ ,  $n=56$ ) was obtained. There was a 25-fold increase in median level of androstenedione in untreated or poorly controlled CAH patients. The measurement of androstenedione levels can thus be used instead of 17-OHP for monitoring therapeutic control in CAH patients since there is less physiological variations among normal population groups and no marked diurnal variation.

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