

Usefulness and limitations of an in-house direct radioimmunoassay for 17-hydroxyprogesterone in serum

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Abstract

Since conventional radioimmunoassays (RIA) for measurement of 17-hydroxyprogesterone (17-OHP) in serum samples require a laborious solvent extraction step, a direct and rapid in-house RIA was developed for early diagnosis and management of congenital adrenal hyperplasia (CAH). In-house rabbit anti-17-OHP antiserum, tritium labelled 17-OHP and dextran-coated charcoal were used in assay buffer with low pH 5.1 and preheated serum samples. Both inter- and intra-assay CVs were <10% and the sensitivity was 1.2 nmol/l or 12 fmol/tube. Results from the direct assay correlated well with values from an extraction assay, $r = 0.88$ in samples from CAH patients, $r = 0.85$ in adults and children, 0.69 and 0.40 in term and preterm neonates respectively, 0.66 and 0.63 in luteal phase and third trimester pregnancy; $p < 0.001$ in all groups except $p < 0.05$ in preterm neonates. However, results from the direct assay were two to three times higher in serum samples from CAH patients, normal adults and children, but were five to seven times higher in pregnancy and term neonates and thirty times higher in preterm neonates. The markedly elevated levels measured by the direct assay are probably due to cross-reactivities with water-soluble steroid metabolites such as 17-hydroxyprogrenolone sulphate and dehydroepiandrosterone sulphate (DHEAS). Although the direct assay is only useful as a screening test for preterm babies, it can be used for both diagnosis and monitoring of treatment of CAH in all other age groups.

Keywords: 17-hydroxyprogesterone, radioimmunoassay, congenital adrenal hyperplasia (CAH)

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an inherited disorder caused by an enzymatic defect in adrenal steroidogenesis, leading to deficient synthesis of cortisol and/or aldosterone.^{1,2} The most common form of CAH, accounting for 95% of patients, is due to deficiency of the 21-hydroxylase enzyme (EC 1.14.99.10) which in turn causes the accumulation of the steroid precursor 17 alpha-hydroxyprogesterone (17-OHP) and increased production of adrenal androgens. It is now evident that measurement of 17-OHP in serum or blood spots is the most valuable laboratory tool for diagnosis and assessment of replacement therapy in CAH patients.³⁻⁵ Specific and sensitive methods available for 17-OHP include radioactive, chemiluminescence, fluorescence and enzyme immunoassays.⁶⁻¹² As most assays require prior extraction of serum or blood samples with organic solvents, several direct non-extraction methods have been introduced.^{9,13,14} In contrast to extraction assays, these direct methods are not

only simpler and less laborious but more importantly, suitable for large-scale screening. However, results of samples from premature neonates and amniotic fluid using direct assays may be difficult to interpret due to water-soluble cross-reacting steroid metabolites.¹⁵⁻¹⁸ In this present study, we developed two reliable, simple and rapid in-house radioimmunoassays (RIAs) by direct and extraction methods and used them to evaluate 17-OHP levels in normal neonate, paediatric and adult population groups and in CAH patients.

MATERIALS AND METHODS

CHEMICALS

6-carboxymethylxime-bovine serum albumin (17-OHP-CMO-BSA), Freund's complete and incomplete adjuvant, 17-OHP and all other steroids and their conjugates, activated charcoal Norit A, 2,5-diphenyloxazole (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; and 17 α -hydroxy[1,2,6,7- ^3H] progesterone (specific activity 63 Ci/mmol) from Amersham International Plc, Bucks., UK. Toluene, ethanol, diethyl ether, petroleum ether and acetone were obtained from May and Baker Ltd., UK., BDH Chemicals Ltd, UK., and E. Merck, Darmstadt, FRG.

REAGENTS

Assay buffer: Citric acid (0.025 mol/l, pH 5.1) containing per litre, 2.5 g citric acid, 9.2 g disodium hydrogen orthophosphate, 1 g sodium azide and 1 g gelatin.

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.

17-OHP standards: Standard solutions, 1.66 to 106 nmol/l, were prepared in assay buffer from a 318 $\mu\text{mol/l}$ (105 $\mu\text{g/ml}$) stock solution of 17-OHP in absolute ethanol. Standards were calibrated against a reference 17-OHP standard generously donated by Dr. A.M. Wallace, Royal Infirmary, Glasgow, UK.

Quality control sera: Three in-house quality control serum pools (QCH, QCM and QCL) were prepared by adding 79.4 and 19.8 nmol/l 17-OHP to a normal basal mixed serum pool of 7.8 nmol/l.

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 of age (mean=8.3, SD=2.3) and pubertal Tanner 2-4 male subjects (n=27) were 12-16 years of age (mean=13.3, SD=1.4). Blood samples were also collected from adult males in the afternoon between 1500-1600h. The normal adults (males=69, females=64) were 20-48 years of age (mean=27.6, SD=7.3) and were staff and students of the Faculty of Medicine, UKM; normal third

trimester pregnant women (n=37) were from the Antenatal Clinic, UKM. Blood samples were allowed to clot and the sera obtained were stored at -20°C.

PRODUCTION OF ANTISERA

Rabbits were injected intra-muscularly with a primary dose of 100 μg 17-OHP-CMO-BSA in Freund's complete adjuvant, followed by multi-site subcutaneous booster doses (100 μg) at monthly intervals.¹⁹ The first test bleed was obtained seven to ten days after the third booster. Antisera were frozen and stored at -20°C or lyophilised and stored at 4°C. The titres and specificities of the test bleeds were determined by RIA. The antisera were used without any further purification, but diluted accordingly in assay buffer.

EXTRACTION ASSAY

To each stoppered glass extraction tube, 3 ml 1:1 petroleum ether:diethyl ether was added to 50 μl serum sample or standard in charcoal-stripped serum. The mixture was vigorously vortexed for 10 min and then the tubes were placed into a solid-CO₂-acetone bath to freeze the aqueous layer. The solvent phase was decanted into another glass tube, evaporated by heating and air-blowing, and the residue reconstituted in 500 μl assay buffer in a 60°C water bath for 20 min. Duplicate 200 μl of the extract was put into plastic LP3 tubes which contained 50 μl [^3H]17-OHP tracer (5-6 000 cpm) and 50 μl of antiserum diluted 7000-fold in assay buffer. The mixture was left to react for 1 hr at room temperature (26 - 28°C) and then incubated for 10 min in ice, after which 500 μl DCC suspension was added to all tubes except to the total count tubes. The tubes were centrifuged for 10 min at 3000 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.

DIRECT ASSAY

Duplicate 10 μl serum samples were put into LP3 plastic tubes containing 200 μl assay buffer. The diluted samples were incubated at 60°C for 30 mins and then allowed to cool before addition of

TABLE 1: Cross-reactivity of anti-17-OHP serum

Steroid	% cross-reactivity
17-OH Progesterone	100.0
17-OH Pregnenolone	25.6
Dehydroepiandrosterone sulphate (DHEAS)	4.0
Progesterone	2.5
Pregnenolone sulphate	2.2
17 Dihydroxyprogesterone	2.0
21 Deoxycortisol	2.6
11 Deoxycortisol	1.2
Pregnanedione	1.0
11-OH Progesterone	0.2
21-OH Pregnenolone	0.1
Cortisol, cortisone, corticosterone, DOC	<0.1
Androstenedione, testosterone, DHT, Estradiol, estriol, estrone, pregnanetriol	<0.01

50 ul 17-OHP tracer and 50 ul of antiserum which had been diluted 7000 fold. The mixtures were then subjected to the subsequent steps as described above for the extraction assay.

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

Antiserum specificity: The antibody specificity is summarised in Table 1, the major cross-reactants being 17-OH pregnenolone 25.6%, DHEAS 4.0%, progesterone 2.5%, pregnenolone sulphate 2.2%, 21-deoxycortisol 2.6%, 11-deoxycortisol 1.2%.

Performance of extraction and direct RIA.

Matrix effect: Displacement standard curves from unextracted or extracted buffer-based standard solutions were not superimposable upon extracted serum-based standards. However, in the direct

TABLE 2: Analytical recovery of 17-OHPRIAs

	n	17-OHP (nmol/l)	Recovery (%)
Extraction assay	47	27.6	96.7 \pm 8.3
	14	86.7	90.3 \pm 11.9
Direct assay	17	38.7	100.3 \pm 6.7
	16	98.3	74.9 \pm 5.6

assay, standard curves were superimposable with assay buffer, pH 5.1 but not with assay buffer, pH 7.4. Thus, standards were prepared in charcoal stripped serum for the extraction assay, while buffer-based standards were used for the direct assay.

Analytical recovery and linearity: Recoveries from analysis of QC serum samples were (mean \pm SD) 96.7 \pm 8.3 and 90.3 \pm 11.9 at 27.6 and 86.7 nmol/l for the extraction assays and 100.3 \pm 6.7 and 74.9 \pm 5.6 at 38.7 and 98.3 nmol/l for the direct assays (Table 2). Dilution of CAH serum (n=6) at 1/20 to give 17-OHP concentrations of 16 - 108 nmol/l, yielded recoveries 93.6-101.9 \pm 4.8-12.9% at further dilutions of 1/40, 1/80 and 1/160 for both assays.

Reproducibility: Intra- and inter-assay coefficient of variations (CVs) ranged from 6.9-8.7% and 9.1-10.9% respectively at 17-OHP concentrations of 8.2, 24.0 and 55.2 nmol/l for the extraction assay (Table 3). The corresponding intra- and inter-assay CVs for the direct assay were 4.3-8.5% and 5.0-8.1% at 18.7, 38.4 and 79.0 nmol/l (Table 3). The RIACalc intra-assay precision profiles (n=49) showed that the CVs were $< 10\%$ over the working range of 1.66-106 nmol/l for the extraction assay and 3.31-106 nmol/l for the direct assay.

Sensitivity: The mean minimum detectable concentration (MDC) defined as the dose at 3SDs from zero binding was 0.7 \pm 0.3 nmol/l or 14 \pm 6 fmol/tube (n=49) for the extraction assay. The MDC for the direct assay was 1.2 \pm 0.6 nmol/l or 12 fmol/tube (n=75).

Comparison: The performance of the extraction RIA was further validated using serum samples from UK-EQAS, an external quality assurance scheme. A significant correlation of $r = 0.96$ ($y = 0.99x + 1.81$, $p < 0.001$, $n = 66$) with the all laboratory trimmed means (ALTM) (no. participants = 27) was obtained (Fig. 1). Overall cumulative bias and variability of bias were

TABLE 3: Reproducibility of 17-OHP RIAs

1-OHP (nmol/l)	Precision			
	Intra-assay		Inter-assay	
	n	CV (%)	n	CV (%)
Extraction assay				
8.2	6	6.9	32	10.9
24.0	6	7.5	46	7.9
55.2	6	8.7	27	9.1
Direct assay				
18.7	6	8.5	13	5.0
38.4	6	4.3	65	5.4
79.0	6	7.1	65	8.1

estimated at -1.7% and 13.1%.

Diurnal variation: Serum samples that were collected in the morning (0900-1000h) had 17-OHP levels, 5.4 ± 1.5 nmol/l (extraction assay, n=25) and 14.2 ± 3.8 nmol/l (direct assay, n=30) that were significantly higher ($p < 0.05$ and $p < 0.01$) than serum samples collected in the afternoon (1500-1600h), which were 4.4 ± 1.4 and 11.7 ± 3.4 nmol/l respectively.

17-OHP levels in normal population groups and CAH patients: The normal physiological levels

of serum 17-OHP in various population groups measured by the extraction assay are shown in Fig. 2 and summarised in Table 4. The highest 17-OHP levels were found in third-trimester maternal serum, followed by preterm neonates, term neonates and luteal phase of adult females, while the lowest values were observed in prepubertal children and in females in follicular phase. Significantly higher 17-OHP levels were obtained in preterm neonates ($0.7-36.3$ nmol/l, $p < 0.01$) compared to term neonates ($0.9-18.1$ nmol/l), neonates ($p < 0.001$) compared to children

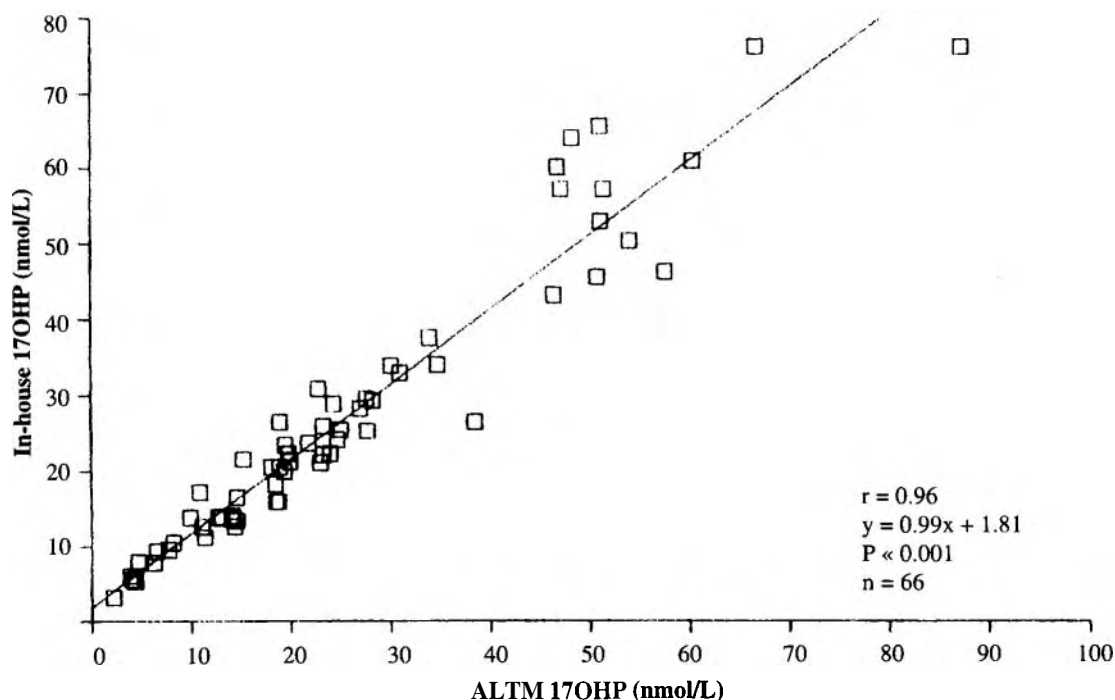


FIG. 1: Performance of 17-OHP extraction RIA in UK-EQAS. Correlation of serum 17OHP values of EQAS samples measured by in-house extraction RIA to all laboratory trimmed means (ALTM) of EQAS.

TABLE 4: Serum 17-OHP levels in normal population groups and CAH patients as measured by extraction RIA

Subject group	n	17-OHP levels (nmol/l)		
		Mean	SD	Range
Neonates:				
Preterm	24	16.0	10.2	0.7 - 36.3
Term	24	9.5 ^a	4.3	0.9 - 18.1
Children:				
Prepubertal	21	1.5 ^b	2.0	0.7 - 6.0
Pubertal males	27	5.3 ^c	2.5	0.7 - 10.4
Adults:				
Males	29	5.2	1.7	1.7 - 8.6
Females:-				
Early follicular	25	3.3 ^d	1.4	0.7 - 6.0
Mid/late follicular	15	3.3	2.5	0.7 - 8.3
Luteal	28	9.3 ^e	2.6	4.2 - 14.4
3rd Trimester	37	31.4 ^{af}	8.8	13.8 - 49.0
CAH patients	23	578(median) ^c		30.9 - 1332

^ap<0.001 vs preterm neonates

^bp<0.001 vs neonates

^cp<0.001 vs prepubertal

^dp<0.001 vs males

^ep<0.001 vs follicular

^fp<0.001 vs luteal, neonates

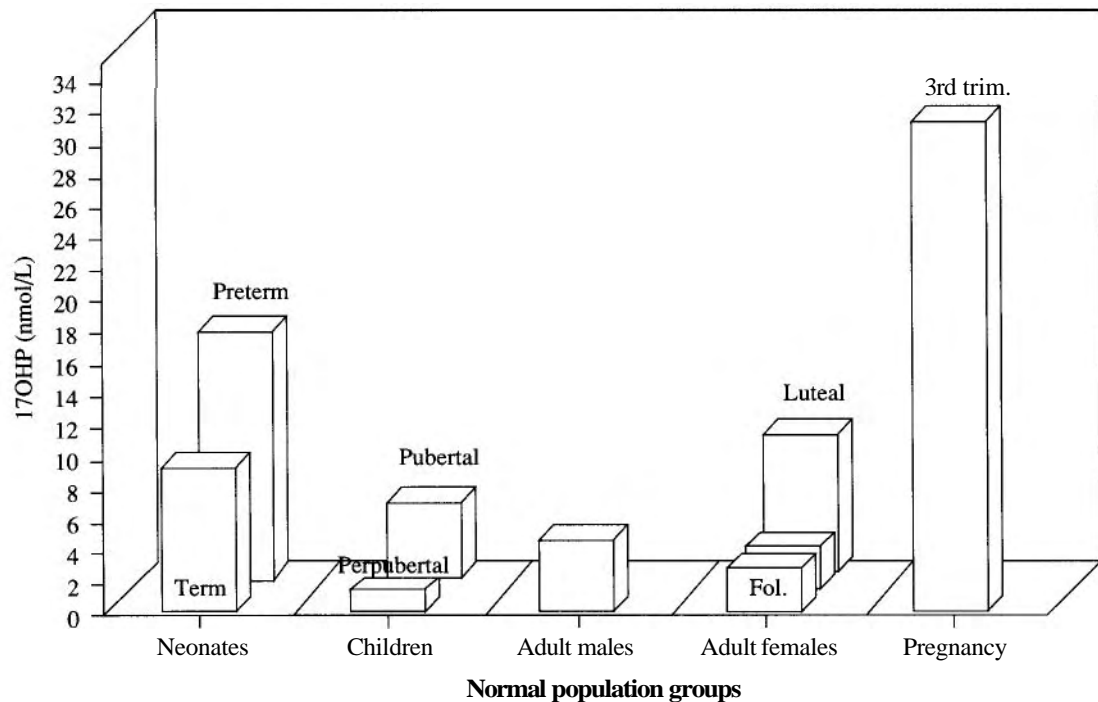


FIG. 2: Mean serum 17–OHP levels of various normal population groups as measured by extraction RIA.

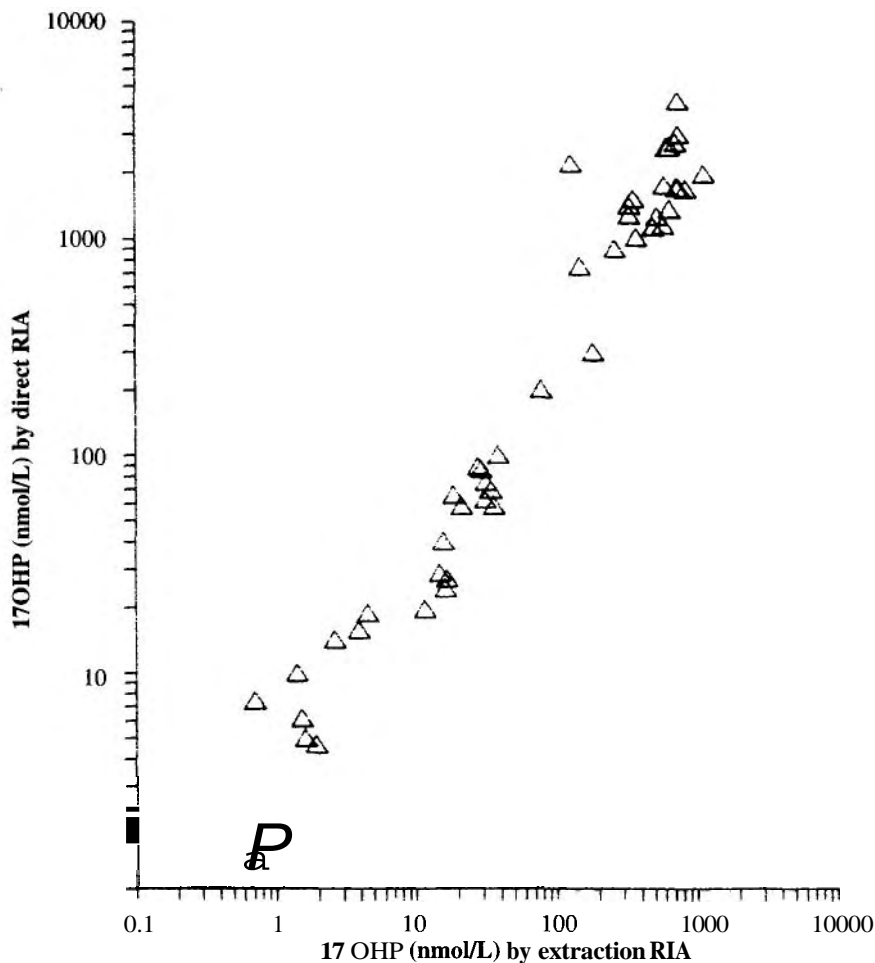


FIG. 3: Correlation of serum 17-OHP levels in CAH patients measured by direct RIA to extraction RIA ($r=0.88$, $y = 3.3x + 16.6$, $p < 0.001$, $n = 55$).

and adults, pubertal males (0.7-10.4 nmol/l, $p < 0.001$) compared to prepubertal children (0.7-6.0 nmol/l), pregnancy (13.8-49.0 nmol/l) compared to luteal phase (4.2-14.4 nmol/l) versus follicular phase (0.7-8.3 nmol/l) ($p < 0.001$). The range of 17-OHP levels in untreated or poorly controlled CAH patients (30.9-1332 nmol/l, median 578 nmol/l) were significantly higher ($p < 0.001$) than the upper normal limit of 6.0 nmol/l for prepubertal children (Table 4).

Regression analyses of serum 17-OHP values determined by both direct and extraction RIAs gave good correlations (Fig. 3 and Table 5). As shown in Figure 3, a highly significant ($p < 0.001$) correlation was obtained for serum samples from CAH patients ($r=0.88$, $y=3.3x + 14.6$, $n=55$). For the various normal population groups, correlation coefficients ranged from 0.57-0.87 ($p < 0.001$) with the exception of $r=0.4$ ($p < 0.05$) for preterm neonates. Although results from the direct assay correlated well with values from

extraction assay, they were approximately two to three, five to seven and thirty times higher in normals and CAH, pregnancy and term neonates, and preterm neonates respectively (Table 5). The highest direct/extraction 17-OHP ratios (range 10.0-73.5, mean 31.2) were found in preterm neonates (Table 5). The various normal reference ranges as determined by the direct assay are shown in Table 6. In contrast to the extraction assay, significantly higher 17-OHP levels were detected in preterm neonates (144-544 nmol/l, $p < 0.001$) compared to maternal samples from third trimester (74-226 nmol/l). There was an overlap in the distribution of serum 17-OHP levels in preterm and term neonates as measured by the extraction assay (Fig. 4a). In contrast, serum 17-OHP levels measured by the direct assay showed two distinct distributions for preterm and term neonates (Fig. 4b); the raised 17-OHP levels remained elevated and distinct from each other up to 16 weeks. Although the

TABLE 5: Correlation of serum 17-OHP levels measured by direct RIA versus extraction RIA

Subjects	Regression analysis				D/E ratio*	
	n	r	y = mx + c	P	Mean	Range
Neonates:						
Preterm	24	0.40	4.4x + 279	<0.05	31.2	10.0-73.5
Term	26	0.69	2.7x + 37.9	<0.001	7.5	4.3-13.7
Children:						
Prepubertal	20	0.87	1.3x + 0.9	<0.001	2.0	0.8- 3.7
Pub. males	26	0.85	1.3x + 4.2	<0.001	2.2	1.3- 3.3
Adults:						
Males	29	0.84	1.2x + 6.4	<0.001	2.4	1.3- 3.4
Females:-						
Foll.	39	0.84	1.5x + 2.5	<0.001	2.5	1.5- 3.7
Luteal	28	0.66	1.1x + 8.3	<0.001	2.1	1.5- 4.3
3rd Trim.	32	0.63	2.9x + 55.0	<0.001	4.9	3.1- 7.2
Patients:						
CAH	55	0.88	3.3x + 14.6	<0.001	3.3	1.6-16.9

D/E ratio is derived from : [17-OHP direct]/[17-OHP extract]

TABLE 6: Serum 17-OHP levels in normal population groups and CAH patients as measured by direct RIA

Subject group	n	17-OHP levels (nmol/l)		
		Mean	SD	Range
Neonates:				
Preterm	29	344	100	144 - 544
Term	27	64 ^a	18.1	28 - 100
Children:				
Prepubertal	21	3.4 ^b	2.1	1.2 - 7.6
Pubertal males	27	11.5 ^c	3.8	3.9 - 19.1
Adults:				
Males	32	14.2 ^d	3.8	6.7 - 21.8
Females:-				
Early follicular	25	7.5 ^e	2.5	2.5 - 12.5
Mid/late follicular	15	7.2	3.8	1.2 - 14.8
Luteal	28	18.7 ^f	4.3	10.1 - 27.3
3rd Trimester	37	150 ^g	38.2	74 - 226
CAH patients	23	928(median) ^c		60.9 - 2100

^ap<0.001 vs preterm neonates

^bp<0.001 vs neonates

^cp<0.001 vs prepuberty

^dp<0.001 vs pubertal males

^ep<0.001 vs adult males

^fp<0.001 vs follicular

^gp<0.001 vs luteal, neonates

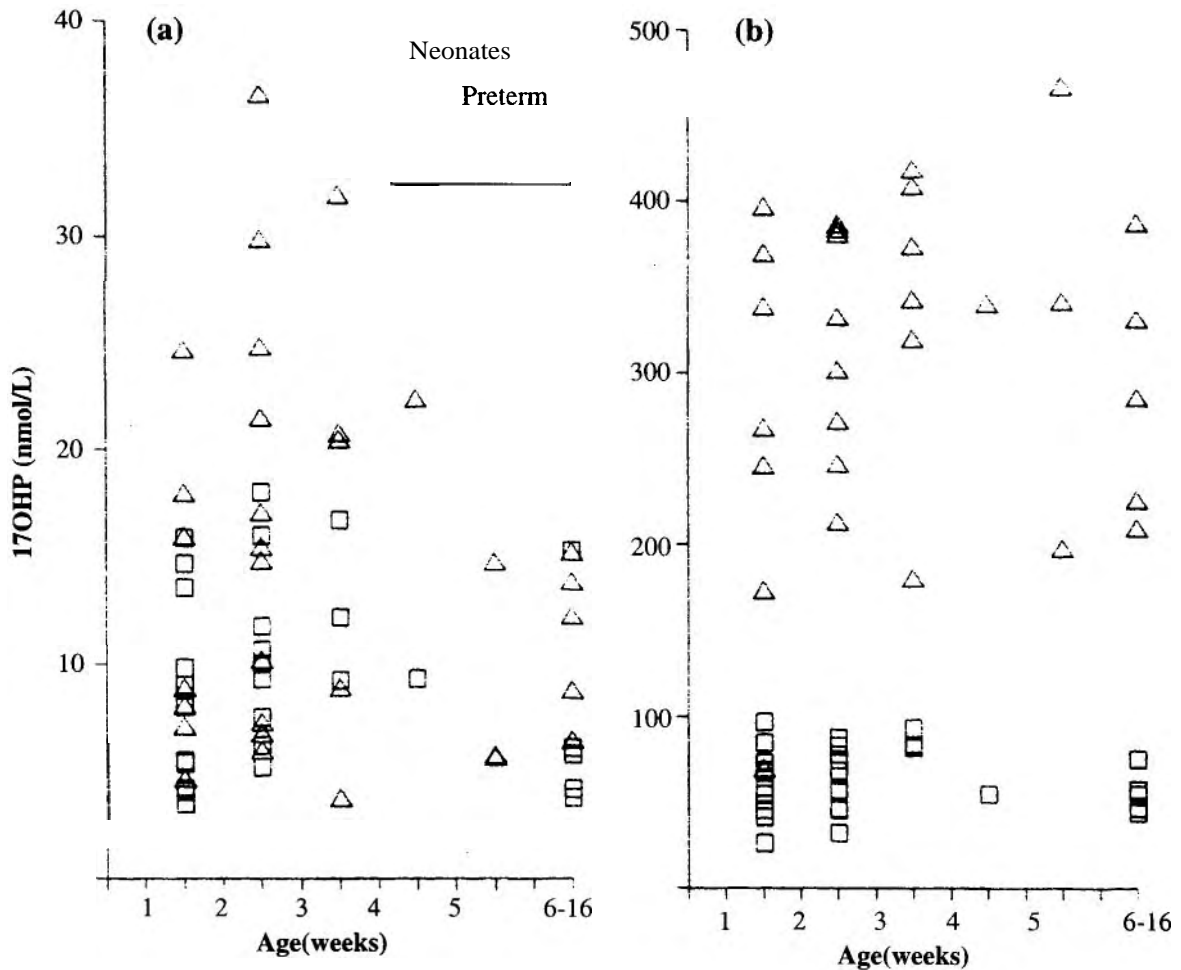


FIG.4: Serum 17-OHP levels in term and preterm neonates of different postnatal ages as measured by (a) extraction RIA, and (b) direct RIA.

higher 17-OHP levels of **preterm** neonates appear to decline with increasing age, there was no correlation ($p>0.1$) with age.

DISCUSSION

Two in-house RIAs for 17-OHP were established and validated. Although the titre (1/42,000) of the in-house antiserum was lower than 1/320,000 and 1/6,000,000 reported respectively by Maeda *et al*⁹ and El-Gamal *et al*,^{11,20} the assay sensitivity of 14 fmol/tube for the in-house RIA is comparable to the range of values of 4.2 to 60 fmol/tube of previous reports.^{7,10-12,21} The antiserum is highly specific with low negligible cross-reactivities with major steroids such as cortisol, progesterone and testosterone. Good results with mean recoveries exceeding 90% were obtained for analytical recovery and linearity measurements of samples spanning the working range for the extraction RIA. For the direct RIA,

there was poor recovery of only 75% at 79.4 nmol/l, although recovery was 100% at the lower concentration of 19.8 nmol/l. In view of this, serum samples with high 17-OHP levels were diluted and re-assayed with values targeted at the lower end of the working range for the direct RIA. The intra- and inter-assay CVs of both the assays were within acceptable limits of <10 and <15%, but the direct assay gave a slightly improved precision (<8%) over the extraction assay. Results from the extraction RIA were 99% of the ALTM of the UK EQAS, and within acceptable control limits of $\pm 10\%$ and 20% for cumulative bias and variability of bias.²²

Results from the extraction assay showed that in the various normal population groups, highest 17-OHP levels were present in third-trimester pregnancy, followed by levels in **preterm** neonates and then term neonates^{9,14,15} while the lowest levels were observed in both prepubertal children

and females in follicular phase. Pubertal males had 17-OHP levels equivalent to adult males which were significantly higher ($p < 0.001$) than those of **adult females in follicular phase** probably due to additional contribution by the testes.²³ In agreement with previous reports,²³⁻²⁵ the level of 17-OHP is a good index of corpus luteum function, being significantly increased ($p < 0.001$) in the luteal phase but not in late follicular phase. The upper limits of the normal reference ranges are in agreement with the reported levels of less than 6 nmol/l for children^{25,26} and 11.3 or 16.8 nmol/l for adults.^{7,10}

The in-house direct assay using low pH and pre-heated serum samples²¹ is cheap and simple as compared to the use of antibody-microcapsules¹³ or Danazol.⁸ However, comparison of serum 17-OHP levels measured by the direct assay with values from the extraction assay showed that the mean levels were two to three fold higher in CAH patients and in normal children and adults. The direct assay also gave mean results that were five to seven fold higher in pregnancy and in term neonates but more than thirty fold higher in preterm neonates. In contrast to the results from the extraction assay, 17-OHP levels in preterm neonates exceeded those in pregnancy and were distinctly separate from term neonates even up to 16 weeks **postpartum**. These discrepantly high 17-OHP levels measured by the direct assay in serum samples from **preterm** neonates, are consistent with previous reports using either in-house direct assays^{9,12,13} or commercial direct RIA kits.²⁸

Wallace *et al*¹⁵ concluded that the markedly high "17-OHP levels in **preterm** infants could be due to cross-reacting 3-beta-hydroxy-5-ene steroids, while Maeda *et al*⁹ attributed cross-reaction to water-soluble intermediates such as 17-hydroxy-pregnenolone. Although we could not firmly identify the actual steroid(s) which is/are cross-reacting with our in-house antiserum, 17-hydroxypregnenolone sulphate and DHEAS may be involved. In view of the higher serum "17-OHP concentrations measured by our direct assay in the various population groups and especially in premature babies, the direct assay can only be used as a screening test for diagnosis. Separate cut-off limits that are dependent on the specificity of the antiserum used, would have to be determined for term and **preterm** neonates and diagnosis of CAH should be confirmed by the extraction assay.^{9,28} However, the direct assay

may be used for therapeutic monitoring purposes since the **direct/extraction** ratios of two to three in CAH patients were similar to the normal population from both paediatric and adult groups.

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