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SERUM CORTISOL IN ADRENAL HIRSUTISM AS ESTIMATED BY FIVE DIFFERENT METHODS

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Summary—Serum cortisol had been estimated in 152 hirsute women complaining of fertility problems, of whom 36 were subsequently diagnosed as having adrenal hirsutism and 10 as having congenital adrenal hyperplasia (steroid 21-hydroxylase deficiency), using five methods: an in-house tritium radioimmunoassay after extraction with ethanol; the Diagnostic Products Corp. "Coat-a-count" iodinated direct radioimmunoassay; the Pharmacia-LKB "DELFIA" lanthanum-enhanced fluoroimmunoassay; the Amersham "Amerlite" luminescence immunoassay; and the Walker "Synelisa" enzyme-linked immunoassay. Although stripped pool serum samples containing weighed amounts of cortisol produced acceptable values in all assays, the patient samples showed a number of high results, much greater than the accepted normal upper limit of 250 ng/ml (25 μg/dl, 690 nmol/l). This was especially so in 21-hydroxylase deficiency, when cortisol values should be very low. Only the luminescence and iodinated assays produced very low values after dexamethasone suppression. After the outliers had been excluded, only the iodinated assay showed a good statistical agreement with the more elaborate tritium assay. The most specific assay was the luminescence method, which produced generally lower results in most cases. This was selected as the new routine method. The unreliable cortisol results in adrenal hirsutism are attributed to high cross-reaction of the antiserum in each of the assays with 17-hydroxyprogesterone, progesterone and 21-deoxyderivatives of cortisol and deoxycorticosterone. In general, all standard and commercially available cortisol assays appears to be unsuitable for cortisol estimation in 21-hydroxylase deficiency, and probably also for neonates.

INTRODUCTION

The existence of adrenal hirsutism as a diagnosis (or disease) depends on the supposition that the patients do not have Cushing's syndrome: therefore the estimation of serum cortisol is of prime importance in the differential diagnosis of the two conditions. It is believed that patients with adrenal hirsutism have an isolated increase in the production of adrenal androgens without an increase in ACTH and/or cortisol production. During puberty ACTH levels go down slightly and cortisol levels rise slightly, while there is development of the adrenal zona reticularis and the production of massive amounts of dehydroepiandrosterone sulphate (DHEAS) and smaller amounts of other adrenal androgens, namely DHEA, androstenedione and testosterone, that is, the response to ACTH alters [1]. Pubertal acne is the most common and mildest form of adrenal hyperandrogenisation that occurs at puberty and is believed to be due to a specific affinity of the large amounts of DHEAS for the skin follicles [2]. More severe hyperandrogenisation produces hirsutism. This is due to the associated overproduction of larger amounts of testosterone and androstenedione, which are much stronger androgens.

A second form of adrenal hirsutism is due to pituitary Cushing's syndrome (Cushing's disease), which is due to the hypersecretion of ACTH, so causing a hypersecretion of cortisol from the zona fasciculata. This also produces acne and hirsutism through the associated hypersecretion of adrenal androgens from the zona reticularis, indicating that this layer is also under ACTH control.

A third form of adrenal hirsutism is due to the absence of cortisol production in the zona fasciculata due to deficiency of steroid 21-hydroxylase. There is a massive build-up of cortisol precursors, particularly of 17-hydroxyprogesterone and of 21-deoxycortisol and their metabolites [3]. Failure of cortisol negative feedback produces a massive hypersecretion of ACTH and a massive adrenal hyperplasia with the histological appearance of a zona reticularis. Sufficient cortisol for the maintenance of life is then produced as a by-product of the androgen pathway at the expense of producing far too much of the adrenal androgens. The earlier this defect develops,

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the more severe the virilisation, so that the congenital cases have the typical genital changes of congenital adrenal hyperplasia while late onset cases may present only with hirsutism associated with menstrual and fertility problems. Cortisol production is always stated to be deficient in 21-hydroxylase deficiency although it has been difficult to substantiate this from values quoted in the literature.

Serum cortisol has been investigated here with five different antisera designed for use in five different modern cortisol assay systems. Serum cortisol levels in adult women with 21-hydroxylase deficiency have been compared with those from normal women and from women with other forms of adrenal hirsutism.

EXPERIMENTAL

Patients

All 152 female patients were being investigated for menstrual and fertility problems, and showed signs and symptoms of hyperandrogenisation, that is, acne, hirsutism and virilisation in varying degrees. No patient had Cushing's syndrome as judged clinically and as shown by a normal diurnal cortisol variation and by a normal overnight dexamethasone test. Similarly, no patient had Addison's disease: all the low cortisol levels arise from the low midnight value in a diurnal variation test or from suppression with either dexamethasone or prednisolone. Serum cortisol was estimated, originally by the in-house tritium method, as part of the differential diagnosis of hyperandrogenisation into gonadal, adrenal or "mixed" forms by the use of gonadal and adrenal suppression tests. Storage of serum samples at −20°C is known not to affect cortisol estimations [4].

Cortisol radioimmunoassay with tritiated tracer

All in-house tritium assays utilise a protein denaturation step which first releases bound cortisol from transcortin and other cortisol binding proteins. Solvent protein denaturation has been performed with ethanol, methylene chloride or with ethylacetate. Other workers have preferred heat denaturation, for example by heating the serum at 60, 70 or 100°C for 10, 15, 20 or 30 min. The official WHO method of 1978 opted for dilution of 50 μl serum to 1 ml with water, incubation at 60°C for 30 min, cooling, and taking 100 μl portions plus 400 μl assay buffer for direct assay [5]. In this department, this method was found not to be so satisfactory as solvent extraction, probably because the released cortisol dissolved better in the aqueous mileau if a non-evaporating hydrophilic solvent is also present. Accordingly, our method follows that of Abraham *et al.*[6] with minor modifications. To two portions of 100 μl serum (true duplicates) are added 400 μl absolute ethanol with vortex mixing, followed by centrifugation at 500 ***g*** for 10 min at room temperature.

The tracer is [1,2,6,7-^{3}H]cortisol. It is essential to perform an additional tracer purification prior to use in the RIA, for which Celite column chromatography is used. Some radioactivity elutes in every fraction, but most (30–50%) in the 45% solvent mixture, which is stored at −20°C and used for the RIA. Standards are prepared at 5, 10, 25, 50, 100, 250, 500 and 1000 pg/500 μl ethanol, that is, per tube. Internal quality control samples are prepared as zero, low and high pools about every 18 months. Stripped serum, theoretically containing no steroids (the zero pool), is prepared by incubating 3 l of outdated serum from the blood bank with 150 ***g*** "active charcoal" (50 mg/ml, Merck) for 2 h at 46°C in a shaking waterbath. Centrifugation and filtration are then carried out 3–5 times, or more, until no more charcoal fines can be removed from the serum. This recovers about 2 l of stripped serum, into a third of which is weighed (using an electronic ultramicrobalance) dried crystalline cortisol (Schering, Berlin, or Sigma, U.S.A.) to provide a low pool at 30 ng/ml and a high pool at 300 ng/ml. The antiserum is anti-cortisol-3-(O-carboxymethyl)oxime-HSA (cat. No. C-001, Steranti Research Ltd, St Albans, England), one vial of which is dissolved in 1 ml buffer (giving a dilution of about 1:10). Portions of 100 μl are stored at −20°C and before use 15 ml buffer is added to one portion, to give final dilution of 1:1500. This dilution has been found by titration to give a binding of 40–50% in the assay.

For the assay, immediately after centrifugation with ethanol, 15 μl supernatant from each extract is removed and mixed with 1.5 ml WHO buffer. The assay is set up with 800 μl buffer in the total assay counts tubes, 600 μl buffer in the NSB tubes, 500 μl standards and 500 μl serum extract. To all tubes is added 100 μl tracer and then to the standards and unknowns only 100 μl antiserum is added. Incubation is for 16–24 h (overnight) at 4°C. Separation of bound and free is with 200 μl WHO dextran-coated charcoal with mixing [0.625 g Norit A (Serva) plus 0.0625 g T70 dextran (Pharmacia) per 100 ml], incubation is for 20 min at 4°C, centrifugation at 1800 ***g*** for 20 min, and then decantation into glass Packard vials, to which is added 10 ml scintillation fluid (Packard "Instagel"). Calculation is with an automatic cubic spline curve on-line to the Packard liquid scintillation counter.

Alternative assays

Most commercially available iodinated radioimmunoassay methods are now "direct", that is they do not perform a preliminary step to release bound cortisol and do not use solvent extraction. The method examined here was the "Coat-a-count" method from Diagnostic Products Corp. (DPC), Los Angeles, Calif. This is the most common kit in use in Germany at the moment, accounting for about 50 laboratories of the 180 that participate in the German quality control scheme. Tubes were counted in a

Table 1. Summary of methods for serum cortisol as performed

Method	Serum volume	Incubation with tracer and antiserum	Separation of bound and free
Tritium method	2 × 100 μl extraction	16–24 h 4°C	Dextran-coated charcoal, centrifuge 20 min
DPC "Coat-a-count" RIA	2 × 25 μl	45 min 37°C	Decant-coated tubes
Pharmacia-LKB "DELFIA" FIA	2 × 20 μl	2 h RT shaking, wash plate 6 times	Enhancement solution, 5 min RT shaking wait 10–15 min
Amersham "Amerlite" LIA	2 × 50 μl	30 min 37°C on plate shaker	Aspirate, wash, signal reagent, read
Walker "Synelisa" ELISA	2 × 20 μl	30 min RT, wash plate once	Substrate 15 min RT, stop with acid

Diagnostic Products Corp. (DPC), Los Angeles, Calif., U.S.A.; Pharmacia-LKB, Uppsala, Sweden; Amersham International, Amersham, England; Walker Laboratories, Ely, England.

Berthold gamma counter, single detector, and calculated with the on-line automatic cubic spline computer programme.

A number of non-radioactive methods have now been described for the estimation of serum cortisol and three recently commercially available assays have been tested here (Table 1). The Pharmacia-LKB "DELFIA" lanthanum-enhanced fluorimmunoassay (FIA) was performed exactly as in the instructions, with reading of the microtitre plates in the Arcus II fluorimeter provided [7, 8]. The Amersham "Amerlite" luminescence assay (LIA) was performed exactly as in the instructions, with reading of the microtitre plate in the luminescence machine provided. The Walker "Synelisa" colorimetric enzyme-linked immunoassay (ELISA) uses the horseradish peroxidase diphenylamine reaction. It incubated 20 μl serum in microtitre plates with antibody coated to the tips of pins hanging down from the lid. In this way, the reaction was started and stopped in each well simultaneously. The colour was read at 492 nm in a microtitre plate reader ("Titertek", Flow Laboratories).

To enable a comparison to be made between the various methods, the standard curves from the non-radioactive methods were recalculated using the IRMA programme in the gamma counter, after insertion of a value for the missing "total counts". This programme first calculates the maximum binding (B_{max}, B_m) using the iteration of a four parameter logistic equation and then calculates the percentage binding in terms of that of the highest standard (B_n) A typical standard curve for the tritium assay was also recalculated using the RIA programme in the gamma counter, this time calculating in terms of B/B_0.

RESULTS

Assay characteristics

All assays produced good dose–response curves (Table 2). The slope at 50% B/B_0 was steepest with the luminescence method, then with the tritium method. The luminescence method was also the most sensitive, being able to detect 2.4 ng/ml as different from nil, that is, calculated as −3 SD from the zero

Table 2. Assay characteristics

Berthold computer programme	DPC "Coat-a-count" 125-I RIA	Pharmacia-LKB "DELFIA" FIA[1]	Tritium method RIA[1]	Amersham "Amerlite" LIA[1]	Walker "Synelisa" ELISA[1]
% NSB/TC	1.45		5.51		
% B_0/TC	69.59		36.08		
Smoothing factor	0.125	0.125	0.125	0.0125	0.125
Goodness of fit	0.0111	0.0151	0.0072	0.0060	0.0370
Slope at 50% B/B_0	0.8617	0.8413[2]	1.0639	1.2566[2]	0.7709[2]
80% B/B_0 (ng/ml)	9.105	46.91[3]	16.177	6.064[3]	20.05[3]
Min. conc. (ng/ml)	0.000	0.362	0.028	0.000	0.207
Det. limit (ng/ml)	3.502	22.022	7.000	2.412	10.77
Sensitivity (ng/ml)[4]	2	1.8		1.0	2

[1]Recalculated in gamma counter.
[2]Slope at 50% B/B_m for upward dose–response curves.
[3]Value at 20% B/B_n for upward dose–response curves.
[4]Taken from kit literature.
Mol. wt of cortisol = 362; nmol/l = 0.0362 μg/dl.

counts. The iodinated RIA was also very sensitive, being able to detect 3 ng/ml from zero. The fluoroimmunoassay was the least sensitive, detecting only about 20 ng/ml as different from zero. It also read values of less than about 46 ng/ml on the less steep and less accurate part of the curve, that is at values less than 80% B/B_0. The ELISA suffers from the disadvantage that a much smaller number of estimations can be made per plate.

Quality control

The internal quality control pool samples were all extremely satisfactory, producing values that were not statistically different from the theoretical. The zero pool produced values between 0 and 5 ng/ml, the low pool between 31 and 42 ng/ml and the high pool 224 ± 31 (173–287) ng/ml [mean $\pm$ SD (range) $n = 14$]. These same pool samples produced slightly lower results in the iodinated assay and in the fluoroimmunoassay. The standards in the fluoroimmunoassay were confirmed to be accurate in the iodinated assay.

It is therefore surprising that our tritium assay regularly produces results that are 10–50% higher than the set value obtained by gas chromatography–mass spectrometry (GC–MS) in the German external quality control system from Bonn. The same high results are produced by the iodinated assay. This is a general effect. When all the different assays from the 180 participants are considered, the 50% value for the results is always higher than the GC–MS value (information from Bonn). It seems that all those cortisol assays, at present in general use, give results that are too high when compared with GC–MS.

Clinical samples

Serum cortisol concentrations showed considerable variations between the five methods, both for persons with or without hirsutism (Table 3) and for those with steroid 21-hydroxylase deficiency (Table 4). When plotted graphically it was seen that all comparisons showed the existence of several outlier pairs, for which the correlation between methods was very poor. Exclusion of the outliers in regression calculations, and exclusion of all results in which one or both values were less than 50 ng/ml (5 μg/dl) (or less than 150 nmol/l), showed that the correlation between the tritium method and the DPC method was the best (Fig. 1), although there was a wide spread. The other comparisons were less satisfactory. Examination of the figures shows that values below 50 ng/ml were not suitable for inclusion in the mathematical analysis. The tritium method tended to give lower results than the fluoroimmunoassay (Fig. 2), although the agreement between the iodinated method and the fluoroimmunoassay was better (Fig. 3). The luminescence method gave the lowest results of all and there was a large difference from

Table 3. Serum cortisol (ng/ml) in persons with or without hirsutism and without 21-hydroxylase deficiency

Sample No.	Tritium method RIA	LKB-Wallac "DELFIA" FIA	DPC I-125 RIA	Amersham "Amerlite" LIA	Walker "Synelisa" ELISA
1	570	167	514,492*	331	
2	511	369	480		
3	458	158	439	356	
4	346			342	
5	346	245	415	323	
6	454	396	412	425	
7	344			232	
8	262			334	
9	279	137	408	214	
10	381	343	373	392	
11	294	273	366		
12	345	325	360		
13	228	99	352	137	
14	284	432	342	298	
15	218	267	309		
16	219	219	304	154	
17	370	145	299	239	
18	118		293	361	
19	264	340	291	286	
20	267	243	273		
21	181	203	268	301	
22		197	267		
23	238	288	266		
24		264	252		
25	255	297	249		
26	280	226	243	177	
27	202	239	240	141	
28		288	240		
29		146	237		
30	252	510	235		
31	208	336	231	197	
32	249	197	230	128	
33	185	271	229		
34	169	150	228		
35		170	228		
36	192		226		
37	267			181	
38	216	277	217	173	
39	207	273	215		
40		162	214		
41	220		211	158	
42	170	231	209	127	
43	285	149	207	193	
44	242			169	
45	216		206	166	
46	222	122	205	187	
47	192		204		
48	209	94	203		
49	264	131	202	98	
50	209	232	200		
51	198			205	
52	185			157	
53	154	73	195,212*		
54	183	201	194		
55	181		194		
56	269	165	194		
57	199	241	190		
58	197	196	185		
59	160		181		
60	199		181		
60	216		180		
61	136	158	179	66	
62	151			103	
63	202			122	
64	213			138	
65	182			107	
66	194	174	177	132	
67	88		175	111	
68	147	166	175	113	
69	172		176		
70	184	186	171		
71		140	167		
72	142		165		
73		182	164		

—*Continued*

Table 3.
Continued

Sample No.	Tritium method RIA	LKB-Wallac "DELFIA" FIA	DPC I-125 RIA	Amersham "Amerlite" LIA	Walker "Synelisa" ELISA
74	135		158	101	
75	114	141	152	108	
76	163		152	97	
77	161	157	151		
78	326	316	147		
79	185		147	123	
80	160	104	145	135	
81	124	113	144	74	
82	164	216	144		
83	135	195	143	104	
84	191	127	141	95	
85	120	208	140	81	92
86	131	196	139,136*		
87	323		139		
88	175	130	136	97	
99	142	140	131		
90	209	220	131		
91	152		129	91	
92	144	128	126	60	71
93	150	137	125	89	
94	127	90	119	82	
95	130	105	117	78	
96	142		117		
97	120			88	
98	127			85	
99	196			64	
100	121			77	
101	218	164	104	180	
102	116	77	103	69	87
103	116			62	
104	110			29	
105	99			62	
106	90			70	
107	83			46	
108	82	85	103		
109	74		101	51	56
110	88	110	100		
111	123	94	100	57	53
112	135	101	96	80	
113	120	71	94	59	
114	114	82	93	74	
115	114	125	91	39	
116	136		90		
117	159		89		
118	162		88		
119	110	59	88	56	
120	104			44	
121	112			77	
122	95	48	84	57	
123	88		84		
123	319	131	83	157	194
124	85	53	77	70	
125	89		72	107	
126	62	69	68	37	37
127	53	56	22	10	18
128	24	54	23	12	18
129		31	28		
130	59			33	
131	57			35	
		Adrenal suppression with dexamethasone			
132	56	28	60	22	33
133	19		9.2		11
134	25	45	5.6		
135	11	3.0	7.3		
136	13			9	
137	17			10	
138	22			15	
139	19			10	
140	20			13	
141	26			14	
142	15			9	

*Values from two separate assays.

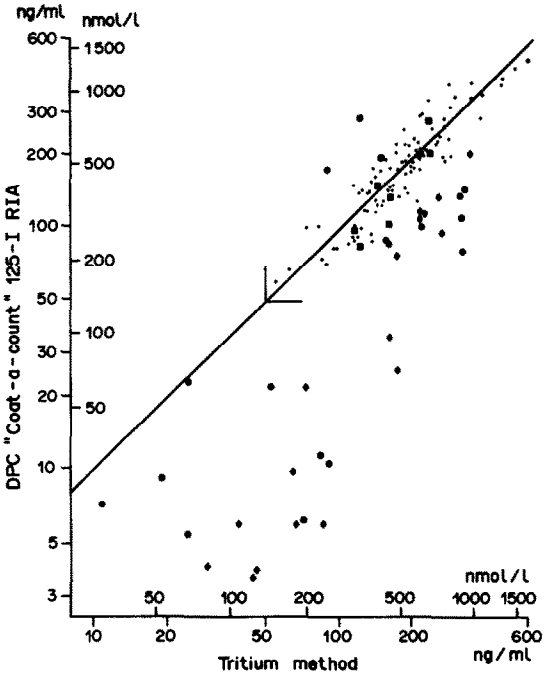

Fig. 1. Cortisol estimation in serum from patients with hirsutism (●●) and steroid 21-hydroxylase deficiency (■◆). Comparison between in-house tritium RIA and Diagnostic Products Corp. "Coat-a-count" iodinated RIA. The 1:1 line is shown. After exclusion of all values below 50 ng/ml (below ∟) and of outliers (●◆), $r = 0.9110$, $y = 7.8 - 0.9623x$ ($n = 99$).

results obtained with the tritium RIA, especially for patients with 21-hydroxylase deficiency (Fig. 4). Comparison of the two best methods showed that the luminescence method still gave lower results and it was necessary to exclude several outliers before a correlation could be calculated (Fig. 5). The ELISA

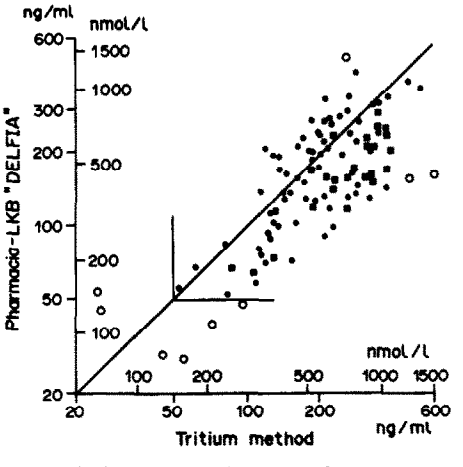

Fig. 2. Cortisol estimation in serum from patients with hirsutism (●○) and steroid 21-hydroxylase deficiency (■). Comparison between in-house tritium RIA and Pharmacia-LKB "DELFIA" fluorescence immunoassay. The 1:1 line is shown. After exclusion of all values below 50 ng/ml (below ∟) and of outliers (○), $r = 0.6544$, $y = 55.4 - 0.5848x$ ($n = 97$).

Table 4. Cortisol in patients with congenital adrenal hyperplasia (21-hydroxylase deficiency)

Patient	17-OHP (ng/ml)	Cortisol (ng/ml) Tritium	"DELFIA"	DPC	"Amelite"	"Synelisa"	Remarks
1 A	162	314	215	116	127	328	Untreated
B	43.7	128	74	22	30	72	DXM
C	36.7	375	172	79			DXM
D	17.1	322	215	112			DXM
E	129	371	253	135	128		Untreated
F	42.0	316	162	139	77		Untreated
G	67.0	387	206		118	180	Untreated
H	46.1	274	174		92	150	Untreated
J	129	371	165		134		Untreated
K	41.9	323	161		93		
L	66.8	326			139	261	
2 A	134	255	158	104	94	246	Untreated
B	52.3	189	120	84	63		Untreated
C	155	257	220	115	88	251	Untreated
3 A	24.7	225	147	148	113		Untreated
B	31.1	131	115	98	67		DXM
4 A	225	211	161	36	58	204	Untreated
B	250	184	173	26	65	248	Untreated
C	27.7	45	29	4	14	38	DXM
D	65.6	107	65	10	28		
5 A	92.9	87	67	6	27		DXM
B	40.8	71	39	6	21	41	DXM
6 A	125	348	261	98	212	377	Untreated
7 A	10.2	229	151	198	168		Untreated
B	12.4	309	228	286	226		Untreated
8 A	61.6	341	298	207	230	240	Untreated
9 A	15.9	224	229	214	158		Untreated
B	11.8	323	161		100		PRED
C	4.73	209			87		PRED
10 A	111	483			265		

DXM = treated with dexamethasone; PRED = treated with prednisolone.

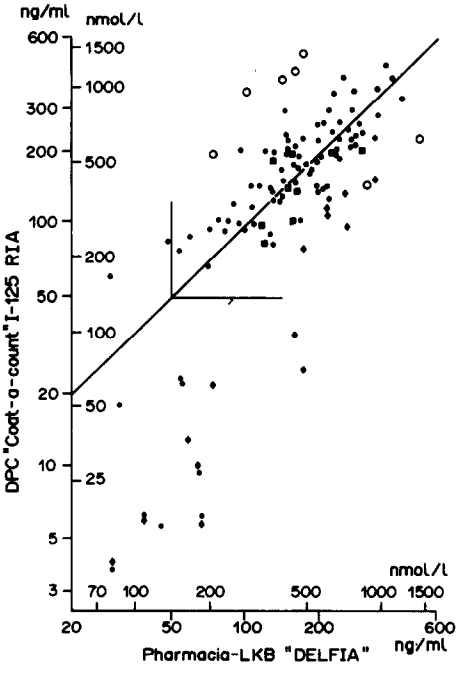

Fig. 3. Cortisol estimation in serum from patients with hirsutism (● ○) and steroid 21-hydroxylase deficiency (■ ◆). Comparison between Pharmacia-LKB "DELFIA" fluorescence immunoassay and Diagnostic Products Corp. "Coat-a-count" iodinated RIA. The 1:1 line is shown. After exclusion of all values below 50 ng/ml (below ⌊) and of outliers (○ ◆), $r = 0.7858$, $y = 37.2 - 0.8348x$ ($n = 85$).

was unsatisfactory, because of the high values produced in patients with 21-hydroxylase deficiency. In every case, addition of the outliers to the regression analysis made the comparisons unsatisfactory. It is clear that the outliers do not belong to the same series as the main results. The luminescence method was selected as our new routine method.

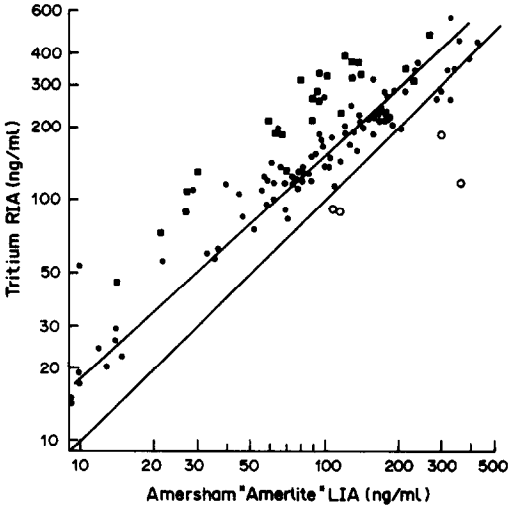

Fig. 4. Cortisol estimation in serum from patients with hirsutism (● ○) and steroid 21-hydroxylase deficiency (■). Comparison between Amersham "Amerlite" luminescence immunoassay and in-house tritium RIA. The 1:1 and the experimental lines are shown. After exclusion of outliers (○), $r = 0.9139$, $y = 46.1 - 1.0178x$ ($n = 88$).

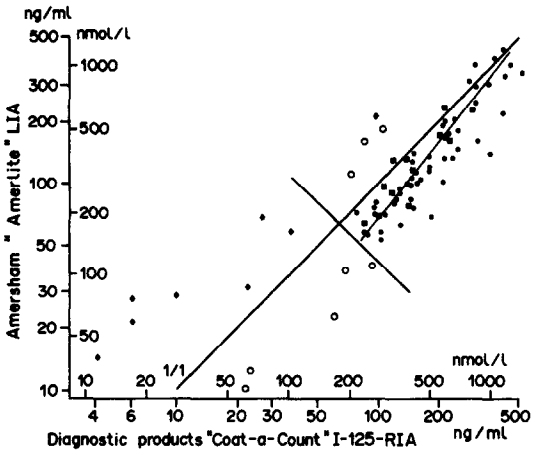

Fig. 5. Cortisol estimation in serum from patients with hirsutism (●○) and steroid 21-hydroxylase deficiency (■◆). Comparison between Diagnostic Products Corp. "Coat-a-count" iodinated RIA and Amersham "Amerlite" luminescence immunoassay. The 1:1 and experimental lines are shown. After exclusion of all values below the cross-line and of outliers (○◆), $r = 0.8757$, $y = -9.4 - 0.8044x$ ($n = 64$).

DISCUSSION

It is clear from the Bonn external quality control samples that it is difficult to obtain "correct" values for serum cortisol with existing assays. The discrepancies between the methods tested here can be attributed to discrepancies between the specificities of the antisera (Table 5). The most specific antisera appear to be in the luminescence and iodinated methods, as these assays gave much lower cortisol values for serum samples in which cortisol production was suppressed with dexamethasone. This accounted for the wide differences in values below 50 ng/ml (or below 150 nmol/l). At this low end of the range it is assumed that the values from the luminescence and iodinated methods are correct. Values as low as 3 ng/ml are achieved with the iodinated method when with the tritium method values only as low as 30 ng/ml were achieved. This raises the problem of the limit to be set for oversuppression. Normal cortisol levels are usually considered to be 50–250 ng/ml (5.0–25.0 μg/dl, 138–690 nmol/l), although the fluoroimmunoassay gives the slightly higher values of

Table 5. Selected cross-reactivities of the cortisol antisera as supplied by the manufacturers

Steroid	Steranti [^{3}H]RIA	Pharmacia "DELFIA" FIA	DPC "Coat-a-count" RIA	Amersham "Amerlite" LIA	Walker "Synelisa" ELISA
Cortisol	100	100	100	100	100
Cortisol 21-glucuronide				<0.3	
Cortisol precursors and their metabolites					
21-Deoxycortisol	50.8				
11-Deoxycortisol	15.3	14.2	0.25	<2.8	10
17-Hydroxyprogesterone	19.5	4.6	0	<0.6	
21-Deoxycortisone			0.04		
Progesterone	2.4	0.7	0.15	<0.2	
Pregnenlone	<0.6				
20α-Dihydroxyprogesterone	<0.6				
Pregnanediol				<0.1	
Cortisol metabolites					
Tetrahydrocortisol			1.0	<0.5	
β-Cortol				<0.1	
20α-Dihydrocortisol				13.1	
20β-Dihydrocortisol				<2.6	
5β-Dihydrocortisol				6.4	
Cortisone		4.3	0.6	<2.6	8.0
Tetrahydrocortisone			0.01	<0.1	
20α-Dihydrocortisone				<0.5	
20β-Dihydrocortisone				<0.5	
5β-Dihydrocortisone				<0.5	
Aldosterone precursors and their metabolites					
Aldosterone				<0.1	0.8
Corticosterone	2.8	1.2	1.4	1.6	9.5
Deoxycorticosterone	<0.6	1.1	1.5	<0.3	2.5
DOC glucuronide				<0.3	
11-Dehydrocorticosterone		1.2			
Adrenal androgens					
Dehydroepiandrosterone		0.03			
Testosterone	<0.6			<0.3	
Androstenedione	<0.6			<0.2	
Epiandrosterone				<0.1	
Synthetic glucocorticoids and mineralocorticoids					
Dexamethasone		2.4	0.53	<0.3	<0.1
Prednisolone	15.3	21.9	46	25.6	18.0
Prednisone	2.0	5.2	3.1	<2.6	
Spironolactone				<0.1	
Canrenone				<0.1	
Fludrocortisone				<2.6	

Steranti Research, St Albans, England; Pharmacia-LKD, Uppsala, Sweden; Diagnostic Products Corp., Los Angeles, Calif., U.S.A.; Amersham International, Amersham, England; Walker Laboratories, Ely, England.

72–308 ng/ml (7.2–30.8 μg/dl, 200–850 nmol/l). In this laboratory, oversuppression was usually considered to be below 30 ng/ml (80 nmol/l) for an early morning sample estimated by the tritium method. This would appear to be correct as the luminescence and iodinated methods show that cortisol production is not completely suppressed at this level and this means that the adrenal glands can recover their function relatively quickly.

Although dexamethasone is always used for a suppression test in suspected Cushing's syndrome, the less strongly active prednisolone is usually used as a therapeutic trial and for maintenance therapy in adrenal hirsutism in this department. Low-dose continuous prednisolone therapy is sufficient to reduce testosterone and DHEAS levels to normal in adrenal hirsutism, while maintaining some residual adrenal cortisol production. If testosterone levels are not decreased to normal, the patient is deemed to have ovarian hirsutism and treated with an ovarian antiandrogen [2]. The cross-reaction of the antiserum in all of the assays is much greater for prednisolone than for dexamethasone. Under prednisolone therapy about 30–40% of the low "cortisol" value is contributed by the drug, so that a higher limit for maximum acceptable suppression is needed.

Many of the outliers in the 50–250 ng/ml range have been shown to be in patients with congenital adrenal hyperplasia. It is considered that the other outliers are due to heterozygotes for this condition who are exhibiting mild adrenal hirsutism. Cortisol levels were invariably apparently elevated with the tritium assay, high normal with the fluoroimmunoassay and again lower but "normal" with the more specific luminescence and iodinated assays. This is contrary to all textbook descriptions of 21-hydroxylase deficiency, in which it is stated that patients are cortisol deficient. It is to be expected that serum levels would be in the range for Addison's disease, that is, just enough to sustain life. The apparently normal or elevated levels found in these assays is undoubtedly due to the cross-reaction with accumulated cortisol precursors. The tritium assay has about a 20% cross-reaction with the large amounts 17-hydroxyprogesterone, while the fluoroimmunoassay has much less and that of the iodinated assay is claimed to be zero. Removal of the cross-reaction with 17-hydroxyprogesterone and progesterone is however not sufficient to reduce cortisol levels to very low values. In the normal pathway of cortisol biosynthesis in the zona fasciculata, 21-hydroxylation of 17-hydroxyprogesterone occurs to form 11-deoxycortisol, which then undergoes 11β-hydroxylation to form cortisol. In the absence of 21-hydroxylase, the 11β-hydroxylation can proceed alone, producing large amounts of 21-deoxycortisol, a substance that sometimes has at least 50% cross-reaction in cortisol assays (Table 5). Levels of 21-deoxycortisol have been shown to be elevated in 21-hydroxylase deficiency [9, 10]. With a specific RIA, levels up to 33 ng/ml have been reported in 21-hydroxylase deficiency, compared with up to 0.2 ng/ml in normal persons and up to 7.0 ng/ml in adrenal hirsutism due to "late onset" 21-hydroxylase deficiency [3]. Most recently, values of 21-deoxycortisol up to 227 ng/ml have been reported in untreated congenital adrenal hyperplasia [11]. Similarly, the large amount of progesterone cannot undergo 21-hydroxylation to form 11-deoxycorticosterone and thence proceed to aldosterone, but 11β-hydroxylation can occur to produce 21-deoxycorticosterone, a substance that is ignored when cortisol assay specificities are measured. Other likely interfering metabolites could be the 18-hydroxy derivatives of 11-deoxycorticosterone and 21-deoxycortisol. Hydroxylation at the 6β- and 12β-positions is also possible [2]. In general, all standard and commercially available cortisol assays appear to be unsuitable for cortisol estimation in 21-hydroxylase deficiency. A similar difficulty has recently been reported for steroid 17α-hydroxylase deficiency [12]. It has been shown here that results can also be very misleading in other cases of adrenal hirsutism.

In addition to adrenal hirsutism, serum cortisol estimations are also known to be unreliable in the newborn infant [13–16]. At birth, about 75% of the adrenal cortex consists of the foetal zone, which produces large amounts of adrenal androgen derivatives. There is increased conversion of cortisol to cortisone, which is the main corticosteroid in foetal blood. The foetal adrenal zone involutes rapidly during the first month of life and more slowly thereafter so that by the end of the first year only the stroma of the degenerated foetal zone remains, while the definitive zone of the cortex is increasing.

In the neonate, as well as in adrenal hirsutism, it seems that the only way to obtain true cortisol values is to perform both extraction of hydrophilic steroids and separation of interfering steroids by paper, thin-layer or column chromatography [16]. Fractionation of the cortisol and 21-deoxycortisol in the solvent extract using columns of Sephadex LH-20 has produced cortisol concentrations of 59 (20–107) ng/ml in 12 cases of 21-hydroxylase deficiency [9], but these values are still probably too high.

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