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Plasma 17-Hydroxyprogesterone Determination with Two Commercial Immunoassays

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Plasma 17-hydroxyprogesterone (17-OH-P) was determined by two commercially available immunoassay kits, a radioimmunoassay (RIA) (OHP-CT, CIS) and an enzyme-immunoassay (EIA) (Serozyme 17 α-OH-progesterone, Serono). The determination by RIA was performed according to two procedures, directly on plasma or on a crude plasma extract, whereas that by EIA used only the second procedure. These determinations were carried out in 27 infants below 1 year of age and in 33 women in the follicular phase of the menstrual cycle. The results were compared to those obtained by an in-home RIA (RIA-FRH) which includes an extraction step followed by chromatography on Sephadex LH 20 column. The levels observed were overestimated by both kits. In infants, interference from 17-hydroxy-pregnenolone (17-OH-5P) sulfate occurred when the RIA (CIS) kit was used directly on plasma samples. Using plasma extracts, 17-OH-5P interfered with EIA (Serono) in the infant group and with the RIA (CIS) in the second group. The two kits do not appear to be adequate for 17-OH-P determination at least in infants and in women in the follicular phase of the menstrual cycle.

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INTRODUCTION

Plasma 17-hydroxyprogesterone (17-OH-P) determination is generally performed for the diagnosis of 21-hydroxylase deficiency and in its treatment [1–3]. Radioimmunoassays (RIA) are commonly used [4] but many non-isotopic techniques have been described. Some of these isotopic or non-isotopic immunoassays are commercially available as ready-to-use kits. The aim of this work was to compare the results obtained by two of these kits: a RIA and an enzyme-immunoassay (EIA), with those of a highly specific RIA (FRH) (reference technique) routinely used in our laboratories. This comparison was carried out in two groups of subjects: infants below 1 year of age and premenopausal women during the follicular phase of the menstrual cycle.

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MATERIAL AND METHODS

Reference technique

The RIA (FRH) considered as the reference technique was a modification of the technique already described [5]. It included an extraction step with diethyl ether followed by chromatography on Sephadex LH 20 column (85 × 200 mm; 10 ml disposable pipettes) in methylene chloride. After an 8 ml wash, 17-OH-P was eluted with the next 4 ml. The eluate was evaporated to dryness and the residue dissolved in 0.5 ml phosphate buffer (pH 6.8). Three aliquots were taken, one of 0.2 ml for estimation of recovery and two of 0.1 ml for RIA as already described [5] except that the tracer was tritiated. Interassay variability was found to be 7.6, 6.3 and 2.2% at the levels of 0.6, 2.0 and 4.0 ng/ml, respectively.

The structurally related steroids, which showed significant cross reaction with the antiserum, were: 17-OH-5P (21.3%), 21-deoxycortisol (4.4%), 11

deoxycortisol (2.9%) and progesterone (P) (1.8%). However, these steroids were not liable to interfere with 17-OH-P assay since they were not eluted in the same fraction from the Sephadex LH 20 column.

Commercial Kits

RIA (CIS)

In this RIA (OHP-CT, CIS Bioindustries International, Gif-sur-Yvette, France) two procedures were applied. The first directly on plasma as recommended by the manufacturer and the second after extraction with diethyl ether.

Procedure without extraction. To the antiserum coated-tubes were added 0.025 ml plasma, serum or standard and 1 ml iodinated 17-OH-P solution. After incubation at 37°C for 2.5 h, the content of all the tubes was discarded and the remaining radioactivity measured in a gamma counter.

According to the manufacturer, intra-assay variability was 8.1, 5.1 and 4.7% at the mean levels of 0.4, 2.5 and 9.9 ng/ml respectively. Inter-assay variability was 9.3, 6.2 and 5.4% at the mean levels of 0.18, 2.7 and 16.7 ng/ml respectively. The detection limit was 20 pg/ml.

Concerning the specificity of the antiserum, 17-OH-5P and 11-deoxycortisol might interfere in the assay (Table 1).

Procedure with extraction. Plasma (0.1 ml) was made up to 0.2 ml with bidistilled water. Extraction was performed with 2 ml diethyl ether. The extract was evaporated to dryness and the residue redissolved with 0.1 ml of the "zero" standard. An aliquot of 0.025 ml was transferred to a coated-tube. The RIA was carried out as described above.

EIA (Serono)

In the EIA tested (Serozyme-17α-OH-Progesterone, Code 022400 Serono Diagnostics), the plasma aliquot was extracted with diethyl ether according to a slight modification of the procedure described in the booklet. Plasma (0.05 ml) was made up to 0.2 ml with bidistilled water and extracted with 2 ml diethyl ether. The extract was evaporated to dryness and the residue dissolved in 0.4 ml phosphate buffer (0.05 M, pH 7.4) containing bovine serum albumin. After addition of 0.1 ml of the 17-OH-P conjugated to horse-radish peroxidase solution and of a polystyrene bead coated with the antiserum, all tubes were incubated overnight at 4°C. The aqueous phase was then discarded and the solid phase washed with 2 × 4 ml bidistilled water. A 2 ml aliquot of the substrate–chromogen mixture was added and the tubes incubated for 30 min at room temperature in the dark. Then 0.2 ml sulfuric acid (2 M) was added. The tubes were shaken and the intensity of the colour measured spectrophotometrically at 492 nm.

Table 1. Cross-reactions (%) of the antisera

Steroid	Serono	CIS
17-Hydroxyprogesterone	100.00	100.00
17-Hydroxypregnenolone	3.80	1.19
Progesterone	1.90	0.10
11-Deoxycortisol	—	1.00
Deoxycorticosterone	—	0.02
Cortisol	0.02	—
Pregnenolone	—	0.01
Pregnenolone sulfate	—	0.01
Corticosterone	—	<0.01
Aldosterone	—	<0.01

According to the cross-reactions reported in the booklet of the kit (Table 1), only 17-OH-5P and P might interfere in the assay. The detection limit was 5 pg. Intra- and interassay variabilities were 8.3 and 12.0%, respectively. The mean recovery of increasing amounts (0.1–6.4 ng/ml) of non-labelled 17-OH-P was 104.2 ± 3.3%.

Determination of 5P, 17-OH-5P and their sulfates

Plasma 5P and 17-OH-5P were determined by RIA techniques including an extraction step followed by Sephadex LH 20 column chromatography [6]. 5P and 17-OH-P sulfates (5PS and 17-OH-5PS) were first hydrolyzed [7], then measured as the corresponding non-conjugated steroids.

Statistical studies

The comparison of the results obtained by the two kits with those of the reference technique (RIA-FRH) was performed according to two methods: linear regression and the ratio method. The differences between the concentrations measured by RIA-FRH (xi) and those by the tested kit (yi), depend not only on yi measurement errors but also on those of xi determination so the method of York was used [8]. It consists of weighting the linear regression by taking into consideration the errors on x and y determinations. The values of b and a of the equation of regression lines:

$$y = (b \pm sb)x + (a \pm sa)$$

were compared to 1 and 0 respectively at $P < 0.05$. When this procedure could not be applied, the equation of the line called major axis was calculated at [9, 10].

The ratio method [11] is based on the distribution of the ratios calculated between the results obtained with the tested technique (Y) and those of the reference technique (X). On the graph showing this distribution, were represented the limits defined at a 95% threshold which corresponded to the variations of the ratios when the reference technique (X) was compared to itself. These limits were calculated from the reproducibility.

In the case of the comparison of 30 determinations, no more than 2 ratio values could be observed above the higher limit and no more than 2 below the lower limit. Partial correlations were calculated according to Sokal and Rohlf [12].

Table 2. Plasma levels (ng/ml) in 27 infants of 17-hydroxyprogesterone (17-OH-P), obtained by different techniques, and of pregnenolone (5P), 17-hydroxypregnenolone (17-OH-5P) and their sulfates

Subject	Age (days)	17-OH-P (ng/ml) M1*	M2A*	M2B*	M3*	5P (ng/ml)	5PS (ng/ml)	17-OH-5P (ng/ml)	17-OH-5PS (ng/ml)
1	2	2.6	15.6	3.5	3.6	3.8	1259	1.8	612
2	2	0.8	9.1	2.0	4.5	6.1	230	14.9	527
3	5	3.1	13.4	3.1	4.5	1.7	2133	4.2	549
4	5	0.5	2.6	0.4	1.0	1.4	124	1.9	199
5	5	0.3	1.8	0.3	0.9	—	—	—	—
6	6	0.4	5.7	1.7	3.6	1.0	301	1.3	294
7	7	0.4	3.3	0.7	0.6	1.0	922	0.6	330
8	9	1.9	7.6	5.3	3.0	0.1	1595	0.1	—
9	12	1.8	9.4	2.5	6.7	2.7	1006	9.4	584
10	13	0.5	2.4	0.7	1.9	1.1	960	0.9	242
11	13	1.0	8.1	1.3	3.4	1.1	715	1.2	415
12	17	1.0	5.7	0.9	2.5	2.6	—	4.5	—
13	18	0.6	3.4	0.8	1.8	1.3	205	2.4	175
14	22	0.9	5.1	0.9	2.1	1.5	144	2.1	99
15	24	0.5	6.5	0.6	1.5	1.7	404	2.2	431
16	30	0.7	6.7	3.0	1.0	1.9	890	2.2	—
17	37	0.7	3.7	1.4	1.9	8.5	1342	4.2	244
18	51	0.2	2.4	0.5	1.3	1.1	117	2.4	90
19	89	1.0	8.5	2.0	5.6	1.6	1165	9.5	791
20	121	0.2	1.8	0.4	1.3	1.1	157	1.9	65
21	150	0.6	4.1	1.2	1.0	3.5	1015	5.5	224
22	246	0.3	0.9	0.6	1.1	1.6	31	1.7	10.6
23	270	1.0	1.1	—	1.9	0.4	24	0.2	—
24	288	0.4	0.2	—	0.4	0.8	—	1.0	—
25	300	0.3	0.6	0.5	0.4	0.1	75	0.2	3.2
26	311	0.1	0.3	0.1	0.5	0.7	41	1.7	5.5
27	365	0.3	1.1	0.4	0.8	0.1	79	0.1	4.8

*M1, RIA (FRH); M2A, RIA (CIS) by the direct procedure; M2B, RIA (CIS) by the extraction procedure; M3, EIA (Serono).

RESULTS

Two subject groups were studied. The first consisted of 27 infants aged below 1 yr who were suspected to have adrenal congenital hyperplasia due to 21-hydroxylase deficiency. The second group consisted of 33 hirsute premenopausal women (age: 18–35 yr) who were screened in the follicular phase for a possible late onset congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

In both groups, 17-OH-P levels were within the range of those observed in age-matched normal subjects [13].

Determination of 17-OH-P in the infant group

The results obtained in this group with the two kits appear in Table 2.

RIA (CIS)

Procedure without extraction (CIS-dir). The levels obtained with this direct procedure (CIS-dir) were markedly higher than those of RIA-FRH. In the equation of the line, called the reduced major axis, calculated because the York procedure could not be applied (Fig. 1):

$$Y\,(\text{CIS-dir}) = (5.44 \pm 0.55)X\,(\text{RIA-FRH}) + (0.40 \pm 0.83),$$

the slope was significantly different from 1 but the intercept with the ordinate axis was not different from 0.

The ratios between the results of the two techniques, CIS-dir and RIA, were very high. In fact, most of the values (21 out of 27) exceeded 5 (Fig. 1).

Procedure with extraction (CIS-ext). The addition of an extraction step to the original procedure, has resulted in much lower levels. In the equation of the regression line calculated between these levels and those of RIA-FRH ($n = 25$):

$$Y\,(\text{CIS-ext}) = (1.30 \pm 0.21)X\,(\text{RIA-FRH}) + (0.31 \pm 0.24),$$

the slope and the intercept with the ordinate axis were not significantly different from 1 and 0 respectively.

The levels were still, however, overestimated, since most of the ratio values (20 out of 25) were above 1, located outside the limits defined above.

It is noteworthy that the differences between the levels obtained by the two procedures with the RIA (CIS) kit tended to decrease with infant age. In fact, the

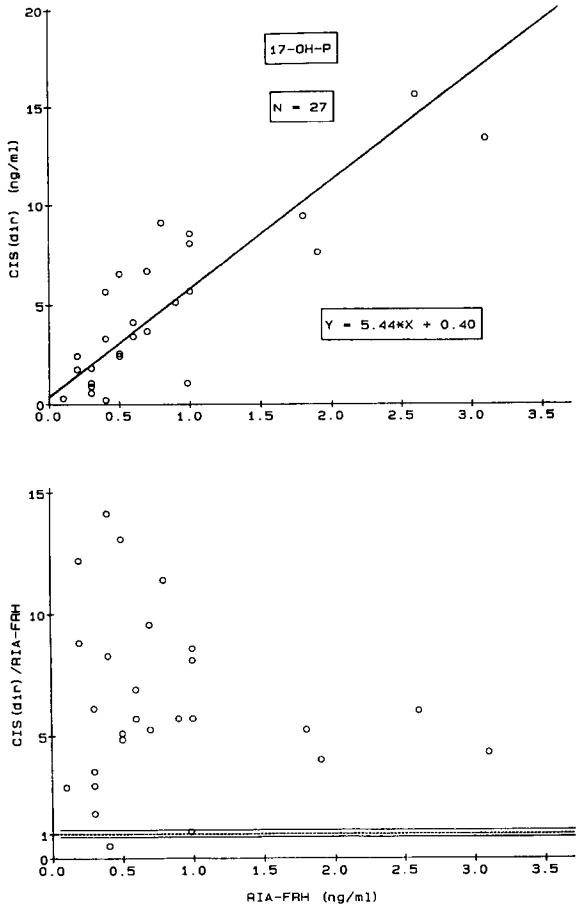

Fig. 1. Comparison of the results obtained in infants with RIA (CIS) kit by the non-extraction procedure [CIS (dir)] with those of the reference technique (RIA-FRH). Upper part: linear regression; lower part: ratio method.

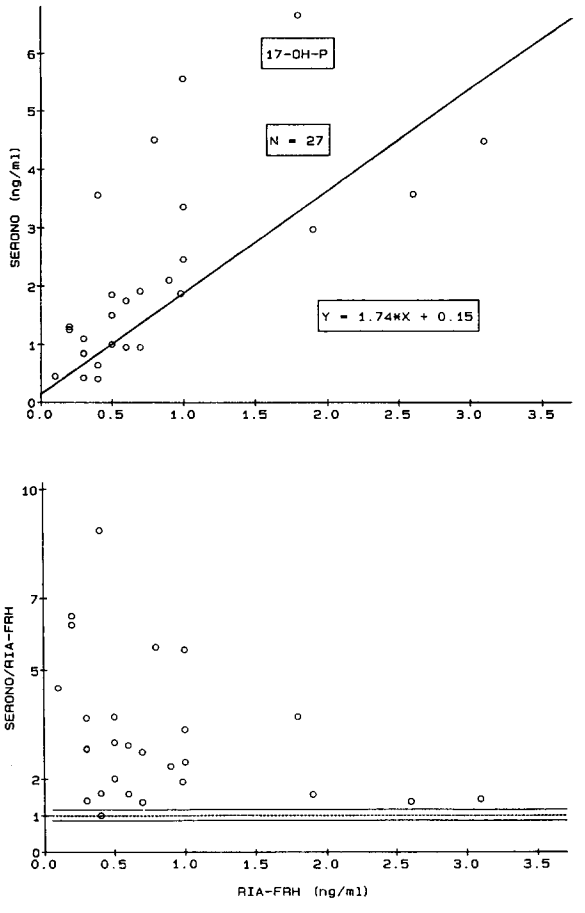

Fig. 3. Comparison of the results obtained in infants with EIA (Serono) with those of the reference technique (RIA-FRH). Upper part: linear regression; lower part: ratio method.

correlation coefficient calculated between these differences and age was -0.54 ($P < 0.01$; $n = 25$) (Fig. 2).

EIA (Serono)

The results observed with this kit were also higher than those obtained with RIA-FRH. The regression line, calculated according to York, had the following equation (Fig. 3):

$$Y\,(\text{Serono}) = (1.74 \pm 0.33)X\,(\text{RIA-FRH}) + (0.15 \pm 15).$$

The slope was still significantly different from 1 but the intercept with the ordinate axis was not different from 0. Moreover, the ratio values were generally higher than 1. In fact, they exceeded 2 in 18 cases out of 27 (Fig. 3).

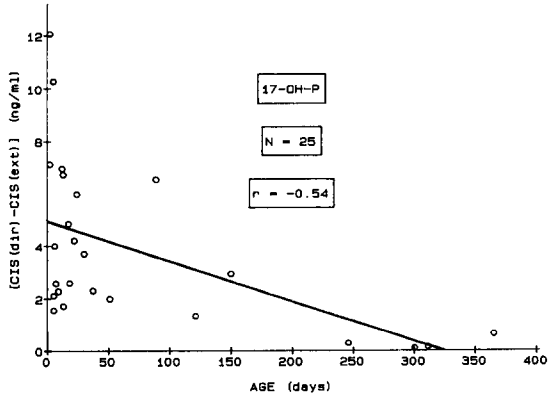

Fig. 2. Correlation of the differences between the levels obtained with RIA (CIS) kit by the non-extraction [CIS (dir)] and the extraction [CIS (ext)] procedures with infant age.

Determination of 17-OH-P in the woman group

RIA (CIS)

Procedure without extraction (CIS-dir). The levels obtained in this group were also overestimated in comparison with the reference technique. However, in the equation of the regression line, calculated according to York (Fig. 4):

$$Y\,(\text{CIS-dir}) = (1.04 \pm 0.38)X\,(\text{RIA-FRH}) + (0.42 \pm 0.14),$$

the slope was not significantly different from 1 but the intercept with the ordinate axis was different from 0. This suggests a constant error. In fact, the majority (26 out of 33) of the ratio values calculated between these

levels and those of RIA-FRH were higher than 2. The discrepancy between the two techniques was more important for the low levels.

Procedure with extraction (CIS-ext). In the equation of the regression line calculated between the results obtained with this procedure and those of RIA-FRH ($n = 33$):

$$Y\,(\text{CIS-ext}) = (1.06 \pm 0.13)X\,(\text{RIA-FRH}) + (0.08 \pm 0.05),$$

the slope and the intercept with the ordinate axis were not significantly different from 1 and 0 respectively.

Conversely, the majority of the ratios (CIS-ext)/(RIA-FRH) was still higher than 1 and 4 values exceeded 2. Thus, the extraction procedure has improved the specificity, yet the disagreements with the reference technique were still present.

EIA (Serono)

The regression line calculated between the levels obtained with this kit and those of RIA-FRH, according to York, had the following equation (Fig. 5):

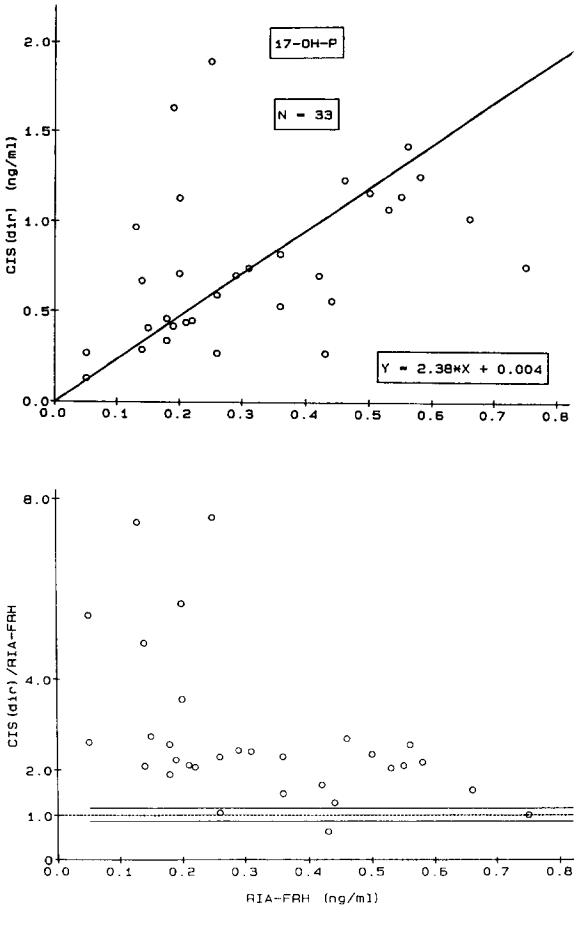

Fig. 4. Comparison of the results obtained in premenopausal women in the follicular phase of the menstrual cycle with RIA (CIS) by the non-extraction procedure [CIS (dir)] with those of the reference technique (RIA-FRH). Upper part: linear regression; lower part: ratio method.

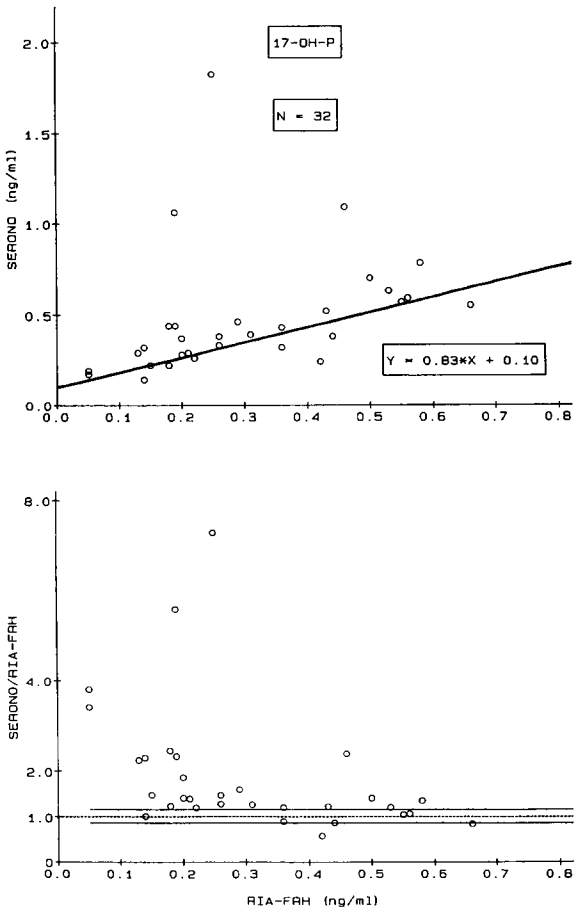

Fig. 5. Comparison of the results obtained in premenopausal women in the follicular phase of the menstrual cycle with EIA (Serono) with those of the reference technique (RIA-FRH). Upper part: linear regression; lower part: ratio method.

$$Y\,(\text{Serono}) = (0.83 \pm 0.15)X\,(\text{RIA-FRH}) + (0.42 \pm 0.14).$$

The slope was not different from 1 but the intercept with the ordinate axis was different from 0. Indeed, this constant error was more obvious with the study of the ratio values. In fact, they exceeded 1 in 26 cases and even 2 in 9 of them. The discrepancies between the two techniques were observed all over the levels range studied, yet they were more important at the low levels.

Plasma levels of 5P, 17-OH-5P and their sulfates

The individual levels observed in infants are grouped in Table 2. As already reported [14, 15], 5PS and 17-OH-5PS levels were inversely and significantly correlated with age, the correlation coefficients being: $r = -0.46$ ($P < 0.05$; $n = 21$) and $r = -0.61$ ($P < 0.01$; $n = 23$), respectively.

In the woman group, the mean level and the range (ng/ml) found were: 0.3 (0.1–1.6), 0.87 (0.1–5.1), 73.9 (41–117) and 4.3 (1.7–8.5) for 5P, 17-OH-5P, 5PS and 17-OH-5PS, respectively. They were comparable to literature data [14–16].

DISCUSSION

Determination of 17-OH-P in infants

In the newborn and the infant, 21-hydroxylase deficiency should be diagnosed promptly so that the adequate therapy be administered [3]. Thus the commercially available kits were designed to obtain the results quickly. This goal is generally attained but simplification of the technique was obtained to the detriment of specificity.

In fact, the specificity of these kits depends entirely on that of the antiserum which was established by the cross-reactions of the structurally related steroids with 17-OH-P. According to these cross-reactions (Table 1) the specificity of both antisera appears to be satisfactory and only 17-OH-5P might interfere particularly when the EIA (Serono) reagents were used. However, in some physiological or pathological states, certain steroids, with a minor cross-reaction, might be present in peripheral blood in such important levels that their interference could not be ignored. This is clearly illustrated in the case of the two tested kits.

Indeed, neither of the reported cross-reactions could explain the results obtained in infants with the RIA (CIS) kit when it was used according to the non-extraction procedure (Fig. 1). The overestimation of the levels, which are about 4-fold higher than those of RIA-FRH, could not be due to the interference of 17-OH-5P which displays the highest cross-reaction.

Including an extraction step in the original procedure produces markedly lower results. Such a difference between the results obtained by a direct and an extraction procedure, has already been reported [17–21] and the fact that this difference decreases with advancing infant age (Fig. 2) is in keeping with Lee and Ellis [21] data. This suggests that a hydrosoluble compound, not extracted with diethyl ether, interferes with RIA (CIS) when the direct procedure is used.

Wallace *et al.* [19] found a significant correlation between the levels of urinary 3β-hydroxy-5-ene steroid sulfates and those of plasma 17-OH-P obtained by either a direct or an extraction procedure. Moreover, this correlation was better when the direct procedure was considered. Thus the interference of 17-OH-5P sulfate (17-OH-5PS) in 17-OH-P determination with non-extraction methods was suggested [17–20]. In fact, though this sulfate cross-reaction was not very important (5.5%) [17] the levels could be very high in infants below 1 yr of age as is the case for 5P sulfate (5PS) [14]. Shimozawa *et al.* [15] showed that 17-OH-5PS levels were very high in newborns, decreasing gradually with age. Recently, the interference of other 3β-hydroxy-5-ene steroid sulfates was reported [22].

The present data, showing simultaneously the levels of 17-OH-5PS, 5PS and those of 17-OH-P obtained by direct and extraction procedures (Table 2) seem to be reported for the first time and confirm clearly that 17-OH-5PS is the main component responsible for the overestimation of 17-OH-P levels when the procedure did not include an extraction step [19, 20, 22]. In fact, 17-OH-5PS levels were significantly correlated with the difference between the 17-OH-P levels obtained by the direct and the extraction procedures ($r = 0.86$; $P < 0.0001$; $n = 21$). Conversely, the correlation between these differences and 5PS levels was not significant ($r = 0.41$; $n = 23$). Partial correlation [12] was obviously calculated because the three parameters, the differences between the two procedures, the levels of 17-OH-5PS and those of 5PS, were inversely correlated with age.

Thus, whereas 17-OH-5PS interference appears clearly, that of 5PS can only be considered as possible. This was confirmed by measuring 17-OH-P in an infant plasma sample without and after adding increasing amounts of 17-OH-5PS (Table 3).

However, the elimination of hydrosoluble components by solvent extraction did not resolve entirely the specificity problem since discrepancies with the reference technique were still present. Since neither 17-OH-5P nor 5P levels were correlated with the differences between 17-OH-P levels obtained by RIA (CIS) with the extraction procedure and RIA-FRH, the overestimation of the 17-OH-P levels could not be due to the interference of these steroids. Thus the interfering compounds are still unknown.

Concerning the EIA (Serono), the obtained 17-OH-P levels were also overestimated in comparison with those of RIA-FRH (Fig. 3) and the differences between the results of the two techniques decreased with advancing age ($r = -0.38$; $P < 0.05$; $n = 26$). According to the cross-reactions of the antiserum, 17-OH-5P might be responsible for these high 17-OH-P levels. In fact, 17-OH-5P concentrations were significantly correlated (partial correlation) with the differences between the results of EIA (Serono) and RIA-FRH ($r = 0.70$; $P = 0.0001$; $n = 26$). No such correlation could be demonstrated with 5P levels.

Determination of 17-OH-P in premenopausal women during the follicular phase

To the best of our knowledge, this is the first study concerning these subjects, the reported data in the literature having been obtained only in infants [18–21].

Table 3. Determination of 17-OH-P in an infant plasma with RIA (CIS) by the direct procedure without and after adding different amounts of 17-OH-5PS ($n = 4$)

17-OH-5PS added (ng/ml)	17-OH-P measured (ng/ml)
0	0.32 ± 0.07
100	1.73 ± 0.15
200	2.76 ± 0.26
500	4.33 ± 0.18

With the reagents of RIA (CIS) kit the results were also overestimated when the procedure did not include an extraction step (Fig. 4). This overestimation was, however, less important than in the case of the infants. This finding confirms that hydrosoluble compounds interference decreased with age as already reported [21]. However, the higher 17-OH-P levels in comparison with those of the extraction procedure could not be due to the interference of either 17-OH-5PS or 5PS. In fact no correlation could be demonstrated between the differences of the two procedures and the levels of either steroid sulfate.

With the extraction procedure, lower results were obtained but they were still higher than those of RIA-FRH. To explain these findings, the differences between the extraction procedure results and those of RIA-FRH were correlated with 17-OH-5P and 5P levels. The correlation coefficient was not significant for 5P ($r = 0.27$; $n = 33$) but was statistically significant for 17-OH-5P ($r = 0.53$; $P < 0.01$; $n = 33$). Thus the interference of 17-OH-5P in the determination of 17-OH-P with RIA (CIS) kit by the extraction procedure seems plausible. This finding could not be predicted in view of the cross-reactions (Table 1) and confirms that the study of the antiserum cross-reactions should not be the unique parameter for the evaluation of the specificity.

In this group of subjects, the 17-OH-P levels obtained by the EIA (Serono) were also higher than those of RIA-FRH (Fig. 5). However, neither 17-OH-5P nor 5P seemed to be implicated in the overestimation of the results since no correlation could be demonstrated between these steroids and the differences calculated between the EIA (Serono) kit results and those of RIA-FRH.

In conclusion, it is not recommended to perform plasma 17-OH-P determination with a non-extraction procedure, not only in infants, as already reported [20], but also in premenopausal women. The extraction procedure allowed elimination of steroid conjugates and non-specific interfering compounds but specificity was still questionable as the present data have clearly demonstrated. In fact, the 17-OH-5P interference could only be eliminated by an adequate chromatographic technique.

Finally, it is more important for the detection of attenuated forms of 21-hydroxylase deficiency to determine 17-OH-P levels with a very specific technique than for the diagnosis of the complete forms. In fact, a false overestimation of the baseline 17-OH-P levels and/or ACTH response might lead to erroneous diagnoses.

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