Original Article

Practicability of Commercial Methods for the Measurement of 17α-Hydroxyprogesterone and Progesterone in Human Saliva

Zur Verwendbarkeit kommerzieller Methoden zum Nachweis von 17α-Hydroxyprogesteron und Progesteron aus Human-Speichel

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Summary: Except for cortisol and DHEAS, commercial assays specifically developed for the analysis of salivary steroids are still not available. Many manufacturers recommend the use of assays for the measurement of plasma also for the analysis of saliva samples. Therefore we evaluated the practicability of 17-hydroxyprogesterone (17OHP) and progesterone (P) RIAs developed for human plasma for the measurement of these steroids in human saliva.

Pooled saliva samples were taken from healthy male individuals and spiked with different concentrations (range 0.2 - 20 ng/ml) of each of the steroids. Using the RIAs as recommended by the manufacturer we achieved good correlations between added and measured steroid concentrations (17OHP: r = 0.986; p < 0.001; P: r = 0.992; p < 0.001) but found 17OHP levels slightly increased (30 – 50%; P/B regression: y = 1.23x + 0.002) and P levels 2-3 fold higher (P/B regression: y = 2.54x + 0.061) than expected. We presume this result is caused by different biochemical conditions in standard material (plasma) and sample matrix (saliva). We therefore adapted the kits by replacing the original plasma standards by in-house saliva standards. After adaptation, 17OHP (P/B regression: y = 1.07x + 0.07; r = 0.998) as well as P (P/B regression: y = 1.05x + 0.07; r = 0.997) were measured in excellent accordance with the amounts added to the saliva samples. No disturbing influences of either dental care (17OHP: p = 0.216; P: p = 0.599) or intake of different foodstuffs (17OHP: p > 0.05; P: p > 0.05) on the reproducibility of the steroid levels could be found. After adaptation, the assays are therefore reliable tools for salivary steroid analysis.

Keywords: Saliva; Radioimmunoassay; Progesterone; 17-Hydroxyprogesterone.

Zusammenfassung: Spezielle Nachweissysteme für Speichelsteroide sind, mit Ausnahme für Cortison, Cortisol und DHEAS, nach wie vor nicht im Handel erhältlich. Viele Hersteller empfehlen die Verwendung ihrer zur Plasmamessung entwickelten Systeme auch für die Bestimmung von Steroiden im Speichel. Daher untersuchten wir RIAs zur Bestimmung von 17-Hydroxyprogesteron (17OHP) und Progesteron (P) aus Plasma auf ihre Verwendbarkeit bei Humanspeichel als Probenmaterial. Ein Speichel-Pool von gesunden Männern wurde mit verschiedenen Mengen der beiden Steroide aufgestockt (Konzentrationsbereich jeweils 0,2 - 20 ng/ml). Unter Verwendung der vom Hersteller vorgeschlagen Arbeitsanleitung ergaben die RIAs gute Korrelationen zwischen zugegebenen und gemessenen Konzentrationen (17OHP: r = 0,986, p < 0,001; P: r = 0,992, p < 0,001). Allerdings waren die 17OHP-Messungen um 30 – 50% erhöht (P/B Regression: y = 1,23x + 0,002), während die Werte für P um das 2 bis 3-fache erhöht waren (P/B Regression: y = 2,54x + 0,061). Wir nehmen an, daß dieses Ergebnis auf die unterschiedlichen biochemischen Bedingungen im Material der Eichkurve (Plasma) und der Probenmatrix (Speichel) zurückzuführen ist. Daher adaptierten wir die Assays, indem die originalen Plasmastandards durch selbst hergestellte Speichelstandards ersetzt wurden. Nach der Adaptation wurden sowohl 17OHP (P/B Regression: y = 1,07x + 0,07; r = 0,998) als auch P (P/B Regression: y = 1,05x + 0,07; r = 0,997) in hervorragender Übereinstimmung mit den dem Speichel zugesetzten Mengen gemessen. Störende Einflüsse auf die Reproduzierbarkeit der Speichelsteroid-Messung konnten weder als Folge des Zähneputzens (17OHP: p = 0,216; P: p = 0,599) noch nach Aufnahme verschiedener Nahrungsmittel nachgewiesen werden (17OHP: p > 0,05; P: p > 0,05). Folglich sind beide Assays nach der Anpassung verläßliche Mittel der Speichelsteroid-Analytik.

Schlüsselwörter: Speichel, Radioimmunoassay, Progesteron, 17-Hydroxyprogesteron.

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Steroid analysis in saliva has become increasingly important over the last years. It is an acceptable alternative to the determination in plasma or serum, especially in newborns [1] and infants [2], because of the stress-free character of even multiple trial sampling [3; 4]. In addition, steroids in saliva represent the non protein-bound, i.e. physiologically active fraction [5]. Many groups use in-house radioimmunological assays (RIAs), adapted for their specific kind of trial-sampling, sample preparation, antibodies, and tracer [6-12]. For clinical studies, the usage of commercial RIAs for the determination of salivary steroids would be advantageous due to increased comparability of the results and easy handling of the assays. Unfortunately, only a few assays are available, mainly for the determination of salivary cortisol [13] and DHEAS [14]. However, to date there are no specific assays for analysis of salivary progesterone (P) and 17α-hydroxyprogesterone (17OHP) commercially available. Both parameters are frequently demanded in therapy control of endocrine diseases, such as congenital adrenal hyperplasia (CAH) [15-17]. Therefore we tested commercial systems for the determination of both steroids in plasma/serum samples as to their suitability for saliva samples and also with regard to a previous adaptation of a commercial plasma assay for the measurement of cortisol in saliva [18].

Material and Methods

Sample Material

For the validation of the assays, pooled saliva from healthy male volunteers was measured directly and after addition of defined concentrations (0.2-20 ng/ml) of 17OHP or P, respectively. For equalisation of the volumes, steroid-free saliva was used. Saliva samples were taken with the polyester Salivette® device (Sarstedt; Nümbrecht, Germany). For the assessment of reference ranges, randomised saliva samples from healthy men (chronological age: 17-58 years; n = 30), women during both follicular and luteal phase of menstrual cycle (age: 13-38 years; n = 28), and prepubertal children (age 3-11 years; n = 42) were measured after adaptation of the commercial RIAs. All samples were taken between 8 and 9 a.m. To study the validity of the assays, saliva samples of children with CAH due to 21-hydroxylase defect (n = 22, 4-13 years) were collected three times a day (7 a.m., 1 p.m., and 7 p.m.) before the intake of a hydrocortisone tablet. In addition, 17 OHP in plasma and saliva samples from patients with CAH (n = 8; 8-15 years) was measured with DSL-6800 before (plasma) and after (saliva) adaptation. An additional experiment was designed to investigate whether dental care and food intake influence the reproducibility of salivary steroid levels. Saliva was collected before and directly after brushing teeth (n = 10) as well as before breakfast, after a cup of milk, after eating a slice of bread, and after chewing a slice of lemon (n = 10). The time interval between the corresponding sample collections was never more than 5 min.

Radioimmunoassays

We used the RIAs DSL-6800 for 17OHP and DSL-3400 for P developed by Diagnostic Systems Laboratories (Sinsheim, Germany). For the measurement of saliva samples, the manufacturer suggested the dilution of the plasma standards of their assays by a ratio of 1:10 to reach the range of concentrations expected in saliva. Data on the assays are presented in Table 1.

RIAs recommended by the manufacturer

Plasma standards in both RIAs were diluted with blank plasma by a ratio of 1:10. The resulting standard curves ranged from 0.01 ng/ml to 20 ng/ml. The tracer volume was kept constant at 500 µl (total capacity ~30,000 cpm). The incubation time remained 1h at 37 ± 2° C.

Table 1 Laboratory data on the radioimmunoassays tested

	17α-hydroxyprogesterone	progesterone
Type of assay	Double antibody	Double antibody
Primary Antibody	Rabbit-anti-17OHP-serum	Rabbit-anti-P-serum
Secondary Antibody	Goat anti rabbit IgG	Goat anti rabbit IgG
Sample-material	serum, plasma	serum
Sample volume	50 µl	50 µl
Sensitivity	2 ng/dl	10 ng/dl
Cross reactions (%)	17OH-Pregnenolone: 3.4 Progesterone: 1.9 Prednisone: 0.11 Dihydroandrosterone: 0.07 11-Deoxycorticosterone: 0.04	5α-Pregnan-3-20-dione: 5.0 11-Deoxycorticosterone: 0.9 17OH-Progesterone: 0.9 5β-Pregnan-3-20-dione: 0.9 20α-Dihydroprogesterone: 0.35 Corticosterone: 0.35 11-Deoxycortisol: 0.27

Adaptation of the RIAs

Plasma standards were replaced by standards in a saliva matrix to completely adjust the assays to biochemical conditions of saliva. Saliva standards were manufactured in steroid-free saliva matrix. For this purpose, a saliva pool was mixed with charcoal (Norit A; Serva, Heidelberg, Germany) and stirred (15 min) to remove components of lower molecular weight, such as steroids. After centrifugation (13,000 U/min, 5 min) and filtration through glass wool (Serva), the saliva matrix was steroid-free (Reference method: GC/MS). The highest standard concentration was based on an ethanolic solution of both steroids (Sigma-Aldrich, Deisenhofen, Germany) and was verified by RP-HPLC. Standards in saliva were produced by serial dilution of stock standards (20 ng/ml) with steroid free saliva. For each of the steroids our standards had a range of 0.01 ng/ml minimum to 20 ng/ml maximum. Steroid-free saliva was used as zero standard. 50 µl of these standards and sample material were required for the assays. The volume of the tracer was halved, from 500 µl to 250 µl (total capacity ~15,000 cpm). The concentration of antibody (AB) solutions was also adapted because of the decreased amounts of steroids. The best results were achieved by diluting the solutions with the primary antibody by a ratio of 1:5 with Na_2HPO_4 buffer (pH 7.4) and the solutions with the secondary antibody a ratio of 1:1 with 4% Polyethylenglycol (Merck, Darmstadt, Germany) in Na_2HPO_4 buffer. The incubation time was increased from 1h at 37° C to 24h at room temperature (20° C). The activity was measured in a gamma counter (Canberra-Packard, Dreieich, Germany) for one minute.

Statistics

The statistical analysis was performed with the EVAP-AK 3.0 (Boehringer, Mannheim, Germany) software for method comparison using the Passing/Bablock (P/B) regression [19] and ANOVA. Box plot graphs represent 25.-75. percentile with whiskers representing 5.-95. percentile.

Results

17α-hydroxyprogesterone

Saliva concentrations measured with the commercial RIA according to recommendations of the manufacturer were 30-50% higher than with our adapted method ($p < 0.001$) (Fig. 1, 2), whereby correlation coefficients between added and measured concentrations were good under both conditions ($r = 0.986$ vs. $r = 0.998$). Accordance between added and measured steroid concentrations was better after adaptation (P/B regression: $y = 1.07x + 0.07$). Intra-assay variance was calculated at 6.2% for 0.03 ng/ml and at 5.9% for 10 ng/ml ($n = 20$), respectively. Inter-assay variance was calculated at 9.5% for 0.03 ng/ml and at 8.6% for 10 ng/ml ($n = 10$), respectively. The determination limit was 0.004 ng/ml, which is an improvement of the data given by the manufacturer (Table 1).

Progesterone

Determination of salivary P as recommended by the manufacturer resulted in 2-3 fold elevated values (Fig. 3) in spite of good correlation ($r = 0.992$). After adaptation, there was excellent accordance between added and measured steroid concentrations (P/B regression: $y = 1.05x + 0.07$; Fig. 4), in addition to even better correlation under adapted conditions ($r = 0.997$; $p < 0.001$). Intra-assay variance was calculated at 7.4% for

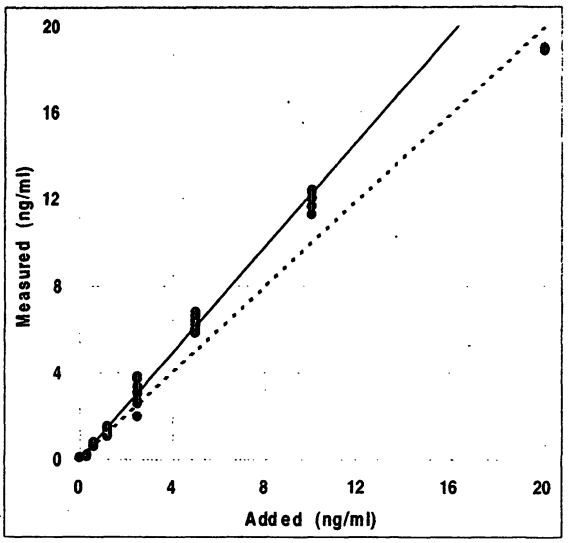

Figure 1 Recovery of salivary 17OHP in spiked saliva samples with DSL-6800 before adaptation: Plasma standards, tracer 500 µl, incubation: 1h at 37°C; P/B regression: $y = 1.23x + 0.002$; $r = 0.986$ ($p<0.001$)

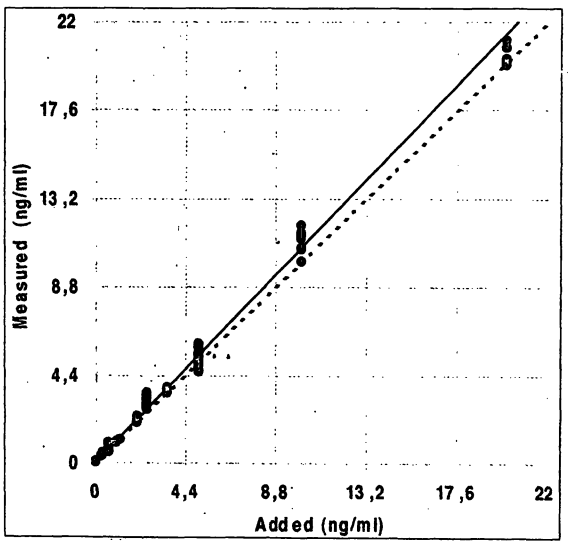

Figure 2 Recovery of salivary 17OHP in spiked saliva samples with DSL-6800 after adaptation: Saliva standards, tracer 250 µl, incubation: 24h at 20°C; P/B regression: $y = 1.07x + 0.07$; $r = 0.998$ ($p<0.001$)

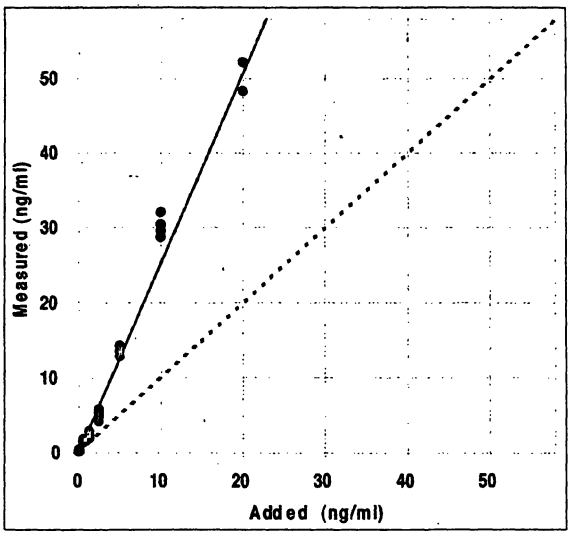

Figure 3 Recovery of salivary progesterone in spiked saliva samples with DSL-3400 before adaptation: Plasma standards, tracer 500 µl, incubation: 1h at 37°C; P/B regression: y = 2.54x + 0.061; r = 0.992 (p<0.001)

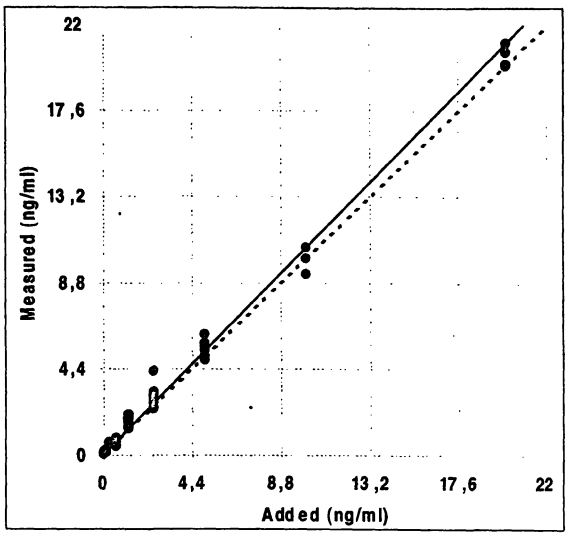

Figure 4 Recovery of salivary progesterone in spiked saliva samples with DSL-3400 after adaptation: Saliva standards, tracer 250 µl, incubation: 24h at 20°C; P/B regression: y = 1.05x + 0.07; r = 0.997 (p<0.001)

0.05 ng/ml and at 5.1% for 8 ng/ml (n = 20), respectively. Inter-assay variance was calculated at 8.5% for 0.05 ng/ml and at 7.7% for 8 ng/ml (n = 10), respectively. The lower determination limit was 0.005 ng/ml. Sensitivity of the antibody used was therefore much better than specified by the manufacturer (Table 1).

Assessment of normal values in healthy volunteers

Reference ranges from healthy men, women during both follicular and luteal phase, and prepubertal children after adaptation of the commercial RIAs are presented in Table 2. Comparison of these values shows good accordance with the ranges from in-house assays described in the literature (Table 3).

Clinical validity of the assays

Validity of the assays is proved by significant differences between follicular and luteal phase P values throughout the menstrual cycle ($p < 0.001$) and by circadian rhythms ($p < 0.001$) of 17OHP in saliva of CAH-patients (Table 2). In comparison to plasma, salivary 17OHP values in CAH patients reached 8-17% of the plasma values (Fig. 5).

Stability of salivary steroid measurements after food intake and dental care

We found no significant changes in salivary steroid values after dental care or food intake, with one exception described below. Levels of 17OHP ($p = 0.23$) and P ($p = 0.60$) were not influenced by brushing teeth

Table 2 Ranges and basic statistics of 17OHP and P in saliva after adaptation in healthy subjects and children with congenital adrenal hyperplasia (CAH) due to a 21 hydroxylase defect (data presented in ng/dl)

		17α-hydroxyprogesterone			progesterone		
	n	mean ± SD	median	range	Mean ± SD	median	range
Female							
Follicular	18	3.8 ± 0.9	3.8	1.7- 5.6	3.2 ± 0.9	3.3	0.9- 4.7
Luteal	27	7.0 ± 2.6	6.2	4.1-12.8	18.4 ± 10.9	15.2	7.0-42.8
Male	42	3.2 ± 0.9	3.2	1.7- 5.1	2.2 ± 0.7	2.2	0.9- 3.2
Children	62	3.0 ± 1.0	1.6	1.0-10.5	3.6 ± 0.8	3.6	0.6- 6.9
CAH	12						
morning		17.2 ± 3.8	18	12.0-22.6	8.0 ± 2.9	7.2	5.6-12.5
noon		7.6 ± 1.7	7.2	5.4- 9.5	5.3 ± 2.3	5.6	2.6- 7.8
evening		4.0 ± 1.2	3.8	2.6- 5.8	2.5 ± 1.2	2.1	1.5- 4.5

Table 3 Laboratory data and reference ranges of in-house immunoassays for P and 17OHP in saliva as presented in the literature. EIA: Enzyme Immunoassay; CLIA: Chemiluminescence Immunoassay; Extr. Extration required; intra: Intra-Assay-Variance; inter: Inter-Assay-Variance; C: Children; F: Female; M: Male; fo: follicular; lu: luteal

Steroid	Ref.	Method	Standard-Range	Sensitivity	CV%	gender	range (ng/dl)
17OHP	[20]	EIA	no data	0.39 pg/tube	5.3-8.8 (intra) 5.3-8.8 (inter)	M/F C	3.3-41.6 1.4-20.3
17OHP	[32]	direct ^{125}I RIA	0.01-2 ng/ml	5 pg/ml	5.3 (intra) 19.5 (inter)	M F	1.6-5.1 2-5.4 (fo) 4.3-10.1 (lu)
17OHP	[7]	Extr.; ^{3}H RIA	0.025-0.25 ng/tube	4 pg/tube	7.1 (intra) 10 (inter)	C	3.0 -50.2
P	[10]	Extr.; ^{3}H RIA	no data	5 pg/tube	6-8 (intra) 10-14 (inter)	F	23.8 ± 1.4 (fo) 47.5 ± 4.0 (lu)
P	[8]	Extr.; ^{3}H RIA	0.01-20 ng/ml	8 pg/tube	5.2 (intra) 9.4 (inter)	F	0.2-0.9 (fo) 3.8-25.4 (lu)
P	[6]	Extr.; ^{3}H RIA	0.01-0.1 ng/tube	7 pg/tube	11 (intra) 8 (inter)	F	<3.1 (fo) 7.2-17.3 (lu)
P	[30]	Extr.; ^{3}H RIA	no data	12.5 pg/tube	13.5 (intra) 7.2 (inter)	F	<5 (fo) 40 ± 11 (lu)
P	[31]	CLIA	0.003 -0.4 ng/tube	1.5 pg/tube	6.8 (intra) 6.9 (inter)	F	5.5 ± 1.4 (fo) 20.7 ± 5.2 (lu)

and the corresponding increase of pH from 7.0 to 7.8. Contamination of saliva with carbohydrates, as expected after eating of a slice of bread, led to a slight decrease of salivary pH, but values of 17OHP ($p = 0.64$) and P ($p = 0.76$) remained almost the same. Fatty acids and proteins from milk did not alter the values of 17OHP ($p = 0.56$), and P ($p = 0.68$), either. The decrease in pH after chewing a slice of lemon increased the steroid values slightly but not significantly for 17OHP ($p = 0.12$) Only the values of progesterone before and after chewing a slice of lemon (Fig. 6) showed a slightly significant increase ($p < 0.05$).

Discussion

The measurement of steroid hormones in saliva is, due to its non-invasive, stress-free method of sample collection, an interesting alternative to the determination in serum or plasma. The non-invasive character is especially important for paediatric endocrinology. The suitability of saliva for steroid measurement in children has been documented in healthy subjects [1; 20], and in pathological conditions such as CAH due to 21-hydroxylase deficiency [16; 21-23]. Since all authors of published work used their own in-house assays, the comparability of results is limited. For example, data

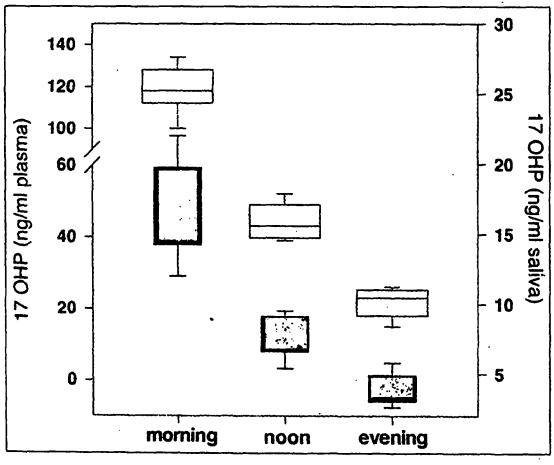

Figure 5 Circadian rhythm of 17OHP levels in plasma □ and saliva ■ of CAH-patients (n=8), measured with DSL-6800 before (plasma) and after (saliva) adaptation

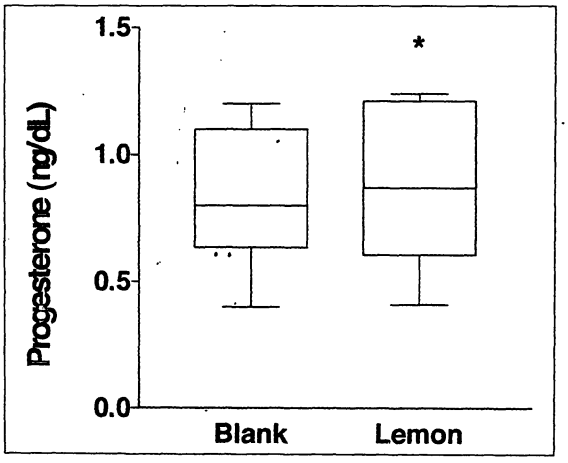

Figure 6 Influence of citric acid (slice of lemon) on salivary progesterone levels.
* represents $p<0.05$

on salivary progesterone in women differ from less than 1 ng/dl [8] to more than 20 ng/dl [10] during follicular phase. This large variability in normal ranges indicates the necessity of uniform measurements. Commercially available kits for the determination of 17OHP and P in saliva would hence be very advantageous, especially for clinical routine or studies. As long as such kits are not available, we were looking for an easy and reliable adaptation of RIAs available for the measurement of these steroids in serum or plasma.

Our results show that the measurement of 17OHP in saliva samples was adequate under conditions recommended by the manufacturer, but determination of P led to clearly elevated results. Possible reasons for this finding could be the difference in binding ability of the progesterone antibody under the dissimilar conditions in plasma and saliva. The pH-dependent affinity of proteins to steroids has been described for the cytosolic receptor protein of glucocorticoids [24] as well as for other plasma proteins [25]. Comparable influences could also apply to steroid-specific antibodies. Whereas pH in plasma ranges from 7.4 to 7.6, we found pH values between 6.9 and 7.1 in saliva samples. However, this difference might be immaterial in view of small sample volumes and much higher volumes of phosphate buffer (pH: 7.4) in antibody solutions. Another reason could be differences in binding affinity of the two steroids to proteins due to their functional groups. Total concentration of proteins with non-specific binding ability for steroids is much higher in plasma than in saliva. For example, albumin concentrations in serum reach >40 mg/ml [26]. In saliva only low concentrations of <2 mg/ml have been found [27; 28]. The hydroxyl group at C17 in 17α-hydroxyprogesterone molecules causes a significant decrease in the binding affinity of this steroid to the glucocorticoid receptor. In contrast, binding affinity of the progesterone molecule to this receptor protein is much higher because of the lack of this functional group [29]. False high levels of progesterone measured in saliva samples using plasma standards could be explained, if these differences also have influence on the binding affinity of the steroids to albumin. In saliva samples there is almost no competition between the highly specific progesterone antibody and the non-specific albumin. In plasma standards however, a certain proportion of progesterone molecules might be bound by the high concentration of albumin and other non-specific plasma proteins. It is therefore not available for binding sites of the antibodies.

Our results show that the introduction of saliva standards instead of diluted plasma standards significantly improves methodical accuracy of the RIAs. After adaptation, we found almost identical values for added and measured steroid concentrations. Our normal data obtained for P with the modified direct RIA were in good accordance with most data reported from other groups using in-house assays [6; 30; 31]. The validity of the assays could be demonstrated as well for physiological hormonal changes (menstrual cycle) as under pathoendocrinological (CAH) conditions. Additionally, the assays showed stability against potential disturbing influences due to food intake and dental care. In summary, it is possible to adapt commercial RIAs developed primarily for plasma/serum for the determination of steroids in saliva. As an extraction step is not required, we have achieved a convenient and reliable instrument for routine clinical conditions.

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