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Salivary Steroids Quantification by HPLC-MS/MS: Comparative Evaluation of Sample Collection Methods for Analysis in Patients with Congenital Adrenal Hyperplasia

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Abstract

Salivary steroids quantification by HPLC-MS/MS: comparative evaluation of sample collection methods for analysis in patients with congenital adrenal hyperplasia.

Steroid hormones analysis in patients with congenital adrenal hyperplasia is usually performed using blood serum or plasma. This approach is not always convenient, particularly when used in children. It is important to note that blood sampling is a stress factor leading to elevated levels of certain steroids. Consequently, non-invasive methods are being developed in order to facilitate the successful assessment of steroid hormone levels across a wide range of matrixes. It is well known that determining saliva steroid hormone levels via high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) is the preferred method for assessing the compensation of congenital adrenal hyperplasia. This technique allows the simultaneous measurement of multiple steroids in a single assay with high accuracy, owing to its minimal cross-reactivity effects.

However, existing saliva collection methods are not always convenient or effective, and the task of selecting a suitable material for sample collection remains crucial, since the commercially available swabs are highly specialized. In this paper we present a comparative study of different saliva collection materials for nine steroids evaluated via high-performance liquid chromatography – tandem mass spectrometry. The present study undertook a comprehensive analysis of an array of materials, specifically including cotton, a biocompatible synthetic polymer, cellulose, and viscose. Concurrently, a rigorous investigation was carried out utilizing biomaterial specimens that were obtained through the application of the free flow collection technique, commonly referred to as the “passive drool” method.

It has been established that the utilization of absorbent materials lowers the concentrations of highly lipophilic steroids. Thus, it has been revealed that the passive saliva run-off into a polypropylene tube technique (“passive drool”) has demonstrated the best approach for collecting saliva samples. Furthermore, a correlation between the hormone levels in saliva and blood serum has been shown in patients with congenital adrenal hyperplasia employing this method of saliva collection.

Keywords: Steroids; Saliva; HPLC-MS/MS, Congenital adrenal hyperplasia; 21-hydroxylase deficiency

Introduction

The Congenital adrenal hyperplasia (CAH) is a group of orphan autosomal recessive disorders characterized by dysfunction of the enzyme or transport protein involved in the cortisol synthesis in the adrenal cortex. Glucocorticoid and mineralocorticoid therapy is the mainstay for CAH treatment [1]. In contrast to other forms of primary adrenal insufficiency, a hallmark of CAH treatment is the administration of glucocorticoids (GCs) at doses above the physiologic requirement to suppress of adrenal stimulation by ACTH. The correct dosage of GCs is critical, as excess GCs assess the risk of obesity and arterial hypertension and leads to decreased growth rates, while GCs deficiency is associated with hyperandrogenism and disease progression. Determination of 17-hydroxyprogesterone (17OHP) and 21-deoxycortisol (21dF) levels as substrates of the enzyme 21-hydroxylase is the primary marker for dose monitoring. However, the enzyme immunoassay (EIA) currently used to determine steroid hormone concentrations does not allow for the simultaneous analysis of multiple components in one assay. The EIA method fails to detect some important steroids, from a diagnostic point of view [2–4]. High performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS) is the preferable method for assessing the compensation of CAH because it allows the simultaneous determination of multiple steroids in a single assay with high accuracy due to the absence of cross-reactivity effects [5,6].

Another challenge in dosage selection is the need for frequent blood sampling to measure hormone levels due to the rapid changes in weight and height during childhood. It should always be kept in mind that blood sampling is a stress factor, leading to elevated steroid levels. In this regard, non-invasive methods are being developed to access the steroid profile [7-10]. Saliva, as a biological material for the determination of steroids by HPLC-MS/MS, is one of them. The potential of using saliva as an alternative substrate has been demonstrated in many studies [5,6,8,11].

Currently, the main technique for saliva collection is the “passive drool” method using special kits (Super Sal, Versi·Sal, Oasis Diagnostic, The Super·Sal, SalivaBio Collection Aid). However, it is difficult to

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collect saliva from young children using this approach, so alternative methods, such as "swabbing" are being developed.

It has been shown previously that the use of Salivette kits with different swab materials (cotton without added any chemical additives , cotton with citric acid and biocompatible synthetic materials) leads, to partial steroid absorption and, therefore, to a change in the determined concentration, regardless of the analytical method used [12–15].

The study investigated other swab materials adapted to a selected set of salivary steroids that could minimize analytical errors. For this purpose, we performed a comparative analysis of the "passive drool" technique into a polypropylene tube (Eppendorf) and saliva collection with swabs made of different materials (cellulose, viscose, cotton without added Salivette® chemical agent, cotton with citric acid and Salivette® bio-compatible synthetic materials) using spiked samples, prepared from the purified saliva. The correlation between steroid concentrations in blood and saliva was also demonstrated.

Materials and Methods

Participants and specimen collection

Samples were collected from eight children undergoing evaluation for hormonally inactive benign thyroid disease (age 6 to 17 years-median 15.5) without adrenal dysfunction (control group).

In addition, samples were collected from 16 patient with confirmed CAH due to 21-hydroxylase deficiency (age 3 to 17 years-median 12) of different sexes who were on daily glucocorticoid (hydrocortisone, prednisolone) and mineralocorticoid (fludrocortisone) replacement therapy.

Saliva samples were collected by the "passive drool" method in the fasting state into polypropylene tubes after rinsing the mouth with water for 10 minutes prior to sample collection. After saliva collection, blood was collected into coagulation t activator tubes, which were then centrifuged to obtain serum. In addition, for comparative analysis of the swabs with the study materials, saliva samples were collected from healthy volunteers (without adrenal dysfunction) using the "passive drool" technique into 50 ml polypopylene centrifuge tubes throughout the day, with the mouth rinsed with water for 10 minutes prior to sample collection. The collected saliva then purified as described below in section 2.3 and applied to test swabs and placed in a polypropylene 1,5 ml tube.

Reagents

Analytical standards for steroid quantification: the following reagents were used as external standards: 11-deoxycortisol (Steraloids, USA), 17α-hydroxyprogesterone (Steraloids, USA), cortisol (Steraloids, USA), cortisone (CIL, USA), 21-deoxycortisol (Steraloids, USA), androstene-3,17-dione (Steraloids, USA), dehydroepiandrosterone (Steraloids, USA), testosterone (Steraloids, USA), and progesterone (Steraloids, USA). The following reagents were used as internal standards: cortisol-d4 (CIL, USA), proges-terone-$^{13}C_3$ (CIL, USA), dehydroepiandrosterone-d_6, testosterone-d_3 (SimSonPharma, India), 17α-hydroxyprogesterone-$^{13}C_3$ (CIL, USA), 11-deoxycortisol-d_5 (CIL, USA), 21-deoxycortisol-d_8 (CIL, USA), and cortisone-d_7 (CIL, USA). Other reagents: methanol (for LC-MS, 99.99%, J.T. Baker, Poland), ethyl acetate (Sigma-Aldrich, for LC-MS, Germany), and zinc sulfate (99,0-100,5%, EMPROVE, Merck, Germany). Deionized water was prepared using the MilliQ Advantage A10 system (Millipore, France).

Sample collection

Salivette® saliva collection containers with a cotton swab without reagent (SARSTEDT AG & CO. KG, Germany), Salivette® Cortisol saliva collection containers with a swab made of biocompatible synthetic material for cortisol levels determination with a label (SARSTEDT AG & CO. KG, Germany), Lemonmoon cellulose sponge (Abrasive Technologies LLC, Russia), viscose wipes (IP Tsvetkov, Russia), 1,5 ml tube (SSIbio, USA), and 50 ml centrifuge tubes (Corning, USA).

Blood samples were collected in BD Vacutainer® SST™II Advance serum separating gel tubes (Becton Dickinson, The Netherlands).

Equipment

The HPLC-MS/MS system used consisted of an Agilent 1290 Infinity II chromato-graph (Agilent Technologies, USA) equipped with a quaternary pump, an autosampler, and a column thermostat, as well as an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Singapore) with a TurboV ionization source with an atmospheric pressure chemical ionization (APCI) probe. Chromatographic separation was carried out on a xSelect HSS C18 2.5 µm, 3.0×75 mm, 2.5 µm particle size column (Waters, Ireland).

Saliva purification and preparation of test samples

To prepare calibration samples for quality control and test samples, we prepared saliva purified from steroid hormones. The purification was performed in two steps: at first, randomized saliva was mixed with activated charcoal, then centrifuged and filtered through a 0.22 µm filter. The filtrate was then passed through cartridges containing C18 sorbent to remove residual steroids. Purity control was performed by HPLC-MS/MS. The criterion for saliva purity was signals below the detection limit (signal-to-noise <3:1) within the expected retention times for each component analyzed. The use of purified saliva instead of water to prepare test samples allowed us to reproduce the conditions of biomaterial collection with all sorption parameters of the steroid substances, which significantly affects the reliability of the results and the reproducibility of sample preparation conditions for calibration and quality control samples.

Three test samples with different counted concentrations of the analyzed steroids (the solutions containing 5% (sample 1), 50% (sample 2), and 150% of steroids (sample 3) of the expected range for each steroid) were prepared from purified saliva (data are shown in Table 1). Test samples were prepared using 150 µl of the appropriate steroid solution and 14,850 µl of purified saliva.

Saliva sample preparation

Sample preparation of all saliva samples was performed according

Table 1: Concentration of steroids in test samples.

Analyte	Sample 1	Sample 2	Sample 3
Cortisone (E), pmol/L	3663	36621	110972
Cortisol (F), pmol/L	1093	10925	33107
21-deoxycortisol (21dF), pmol/L	124	1238	3752
11-deoxycortisol (11dF) , pmol/L	124	1238	3752
Androstenedione (A), pmol/L	92	922	2793
Testosterone (T), pmol/L	51,5	515	1560
17a-hydroxyprogesterone (17OHP), pmol/L	194.5	1945	5894
Dehydroepiandrosterone (DHEA), pmol/L	34.9	349	1059
Progesterone (P), pmol/L	21	210	636

to the method described by R. Jurgens et al. [16]. To 500 µl of saliva, 25 µl of internal standard solution and 900 µl of ethyl acetate were added. After mixing on a shaker, the upper organic layer was separated, evaporated to dryness, and redissolved in 110 µl of 30:70 methanol-water.

Serum sample preparation

Serum samples were prepared according to our previously described method [17]. Briefly, 30 µL of internal standard, 150 µL of 0.1 M zinc sulfate solution, and 200 µL of methanol were added to a 300 µL aliquot of serum. After mixing on a shaker, the sample was centrifuged, and the supernatant was evaporated in a vacuum centrifuge to remove the organic solvent. Next, 500 µl of ethyl acetate was added to the residue, mixed in a shaker, and the upper organic phase was separated and evaporated to dryness. The dry residue was redissolved in 120 µL of 30:70 methanol-water and used for HPLC-MS/MS analysis.

HPLC conditions

The chromatographic separation was performed in a gradient mode using a two-component gradient based on methanol (A) and water (B). The flow rate was set to 0.4 ml/min, the column temperature was 40°C, and the sample injection volume was 80 µl. For the separation a following gradient program was used: the elution started at 50% of A lineary increased from 0 to 6 min to 60% of A, then lineary increased from 6 to 9 min to 90% and then kept isocratic for 2 min. For the re-equilibration, A was reduced to initial conditions in 1 min and kept isocratic for 3 min. Total run time was 15 min.

MS conditions

A triple quadrupole mass spectrometer equipped with an APCI source was applied in positive ion mode, the nebulizer current (NC) was set to 5 uA, the nebulizer gas (GS1) was 50 psi (zero air), the curtain gas (CUR) was 28 psi (nitrogen), and the source temperature was 500°C. Component acquisition was performed in the scheduled MRM mode. The MRM detection window was 90 s, and the target cycle scan time was 1 s. Q1 and Q3 masses were selected by analyzing product ion mass spectra of each standard. The MRM ion optics parameters were selected individually for each component by ramping corresponding parameter (CE, DP, EP, CXP). Quantitative data were selected and processed using Analyst 1.6.3 software (AB Sciex).

Statistical analysis

Statistical analysis was carried out on Python 3.11 using scipy library 1.11.1. Correlations between definitions were assessed with Spearman's rank test

Results

Testing of the cellulose and viscose tampons

To assess the cleanliness of the swabs, purified saliva was applied to an absorbent material, centrifuged at and the resulting sample was further used for sample preparation. The prepared samples were then analyzed by HPLC-MS/MS. The results of the analysis showed the presence of substances in the viscose with retention times, MRM transitions and chromatographic peak ratios of the quantifier to qualifier corresponding to DHEA and progesterone. Therefore, these samples were pre-washed with methanol and water, followed by a purity control.

To test swabs made of cellulose, viscose, and cotton swabs without the addition of a chemical agent Salivette® and swabs made of biocompatible synthetic materials Salivette®, we applied test samples with different analyte concentrations to each swab. After 30 minutes they were centrifuged and used for sample preparation. In addition, to test the ability to use of Eppendorf 1,5 ml tubes as a saliva collection method, 2 ml samples with different steroid concentrations were prepared and used immediately for sample preparation. Saliva samples collected from patients in Eppendorf tubes were used immediately for sample preparation.

The results obtained from all the sample collection and pretreatment methods are shown in Table 2

*E – Cortisone, F – Cortisol, 21dF – 21-Deoxycortisol, 11dF – 11-Deoxycortisol, A – Androstenedione, T – Testosterone, 17-OHP – 17α-hydroxyprogesterone, DHEA – Dehydroepiandrosterone, P – Progesterone

When using swabs, we found a pattern in which the percentage of steroid loss on swabs directly correlated with the lipophilicity of the steroids. The more lipophilic the steroid, the more it was adsorbed to the swab material, thereby reducing the results obtained (Figure 1, Figure 2 and Figure 3).

Cotton swabs without the addition of the chemical agent Salivette® showed the abnormally elevated results for progesterone,

Table 2: The results of determining the content of steroids depending on their concentration and the type of swap used.

% from expected content*		E	F	21dF	11dF	A	T	17-OHP	DHEA	P
Salivette® cotton swab without the addition of a chemical agent	Sample 1	88.5	81.6	43.7	63.2	139.2	113.5	48.5	789.1	462.5
	Sample 2	88.3	85.7	83	68	93.2	70.1	52.4	129.1	62.7
	Sample 3	88.2	86.4	81.8	72.2	92.2	68.5	55.8	73.9	34.2
Salivette® swab made of bio-compatible synthetic materials	Sample 1	96.6	93.8	92.9	85.8	81.8	45	36.9	18	2.2
	Sample 2	99.7	97.8	94.6	85.6	88.5	58.5	60.1	40.2	5.5
	Sample 3	98.5	98.3	96.1	88.5	95.2	61	62.6	43.1	6
Viscose	Sample 1	84.3	85	115.8	76.6	68.5	78	56	70.8	91.3
	Sample 2	86	86.6	85.5	73.8	59.5	60.1	54.7	47.5	13.1
	Sample 3	85.7	87.6	82.7	71.1	57.9	55.4	49.3	43.6	8.6
Cellulose	Sample 1	86.2	85	106.1	75.6	73.1	75.5	66.7	80.1	34.8
	Sample 2	87	86.1	86.1	72.4	59.7	63.3	49.6	58.5	22.1
	Sample 3	88	89.7	86.8	79	70.8	68.6	63.9	66.6	33.2
Eppendorf	Sample 1	99.6	97.3	103.7	103.6	97.9	101.3	101.9	102.9	101.3
	Sample 2	93.1	90.8	94.6	94.5	91.6	94.3	95.5	95.7	96.7
	Sample 3	91.5	90.2	93.2	94.2	94.3	94.7	94.6	90.6	94.4

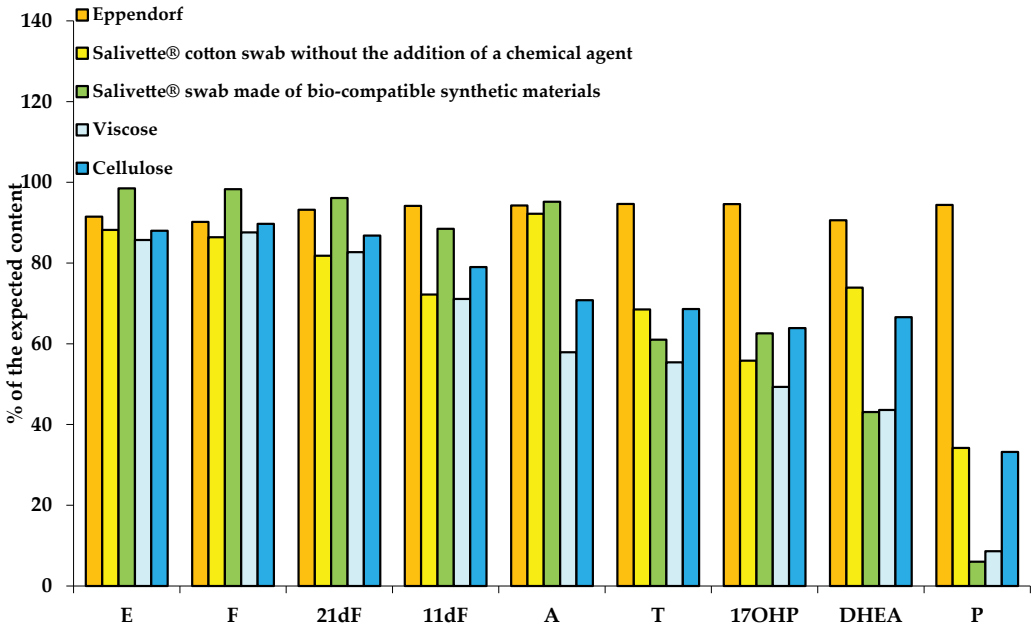

Figure 1: Sample 2 (50% of expected range).

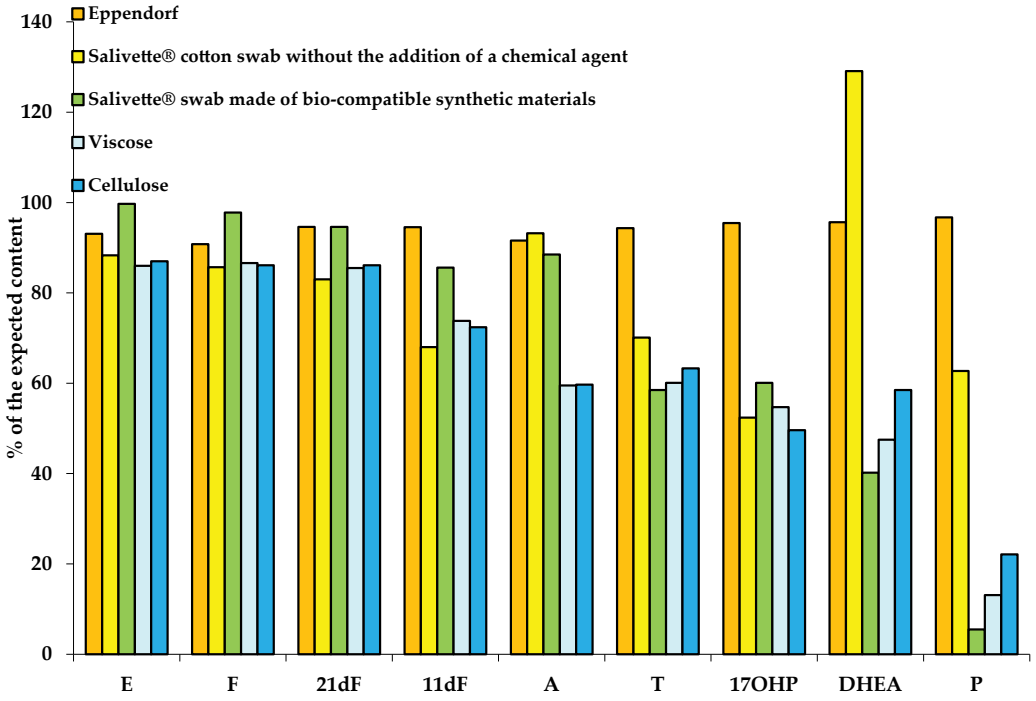

Figure 2: Sample 3 (150% of expected range).

testosterone, DHEA, and androstenedione when we analyzed samples with the lowest steroid concentration (5% of the expected range). To determine the source, we repeated the sample pretreatment and sample preparation using purified saliva. Under the conditions of the method, we detected the presence of substances in a cotton swab under the conditions of the method that have the same retention times and MRM transitions, maintaining the ionic ratio, as the indicated analytes (DHEA, progesterone). The similar trend was observed for all samples tested with a similar swab. However, the deviation is more obvious, in samples with the lowest percentage of the expected ranges.

Correlation between steroid concentrations in blood and saliva.

Analysis of the steroid profile in saliva using the "passive drool" technique in polypropylene 1,5 ml tubes in a group of patients (N=16), showed a positive correlation between serum and saliva in the concentration of androstenedione ($p = <0.001$; $r = 0.94$), 17α-hydroxyprogesterone ($p = <0.001$; $r = 0.85$), cortisone ($p = 0.008$; $r = 0.63$), 21-deoxycortisol ($p = 0.001$; $r = 0.74$), DHEA ($p= 0.023$; $r = 0.57$), and progesterone ($p= 0.025$; $r = 0.57$) (Figure 4).

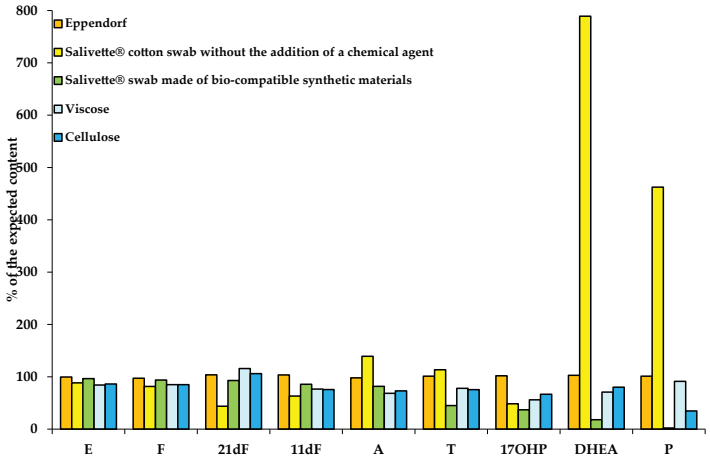

Figure 3: Sample 3 (5% of expected range).

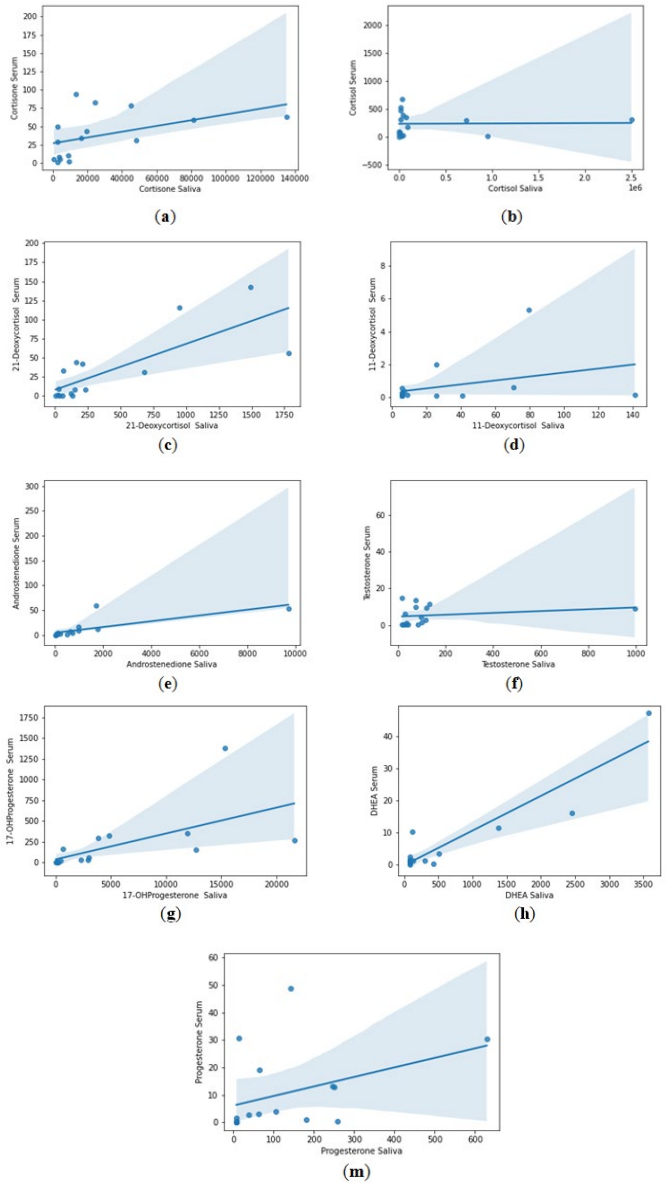

Figure 4: Correlation of steroids between blood and saliva in patients with CAH ((a)-cortisone, (b)-cortisol, (c)-21-deoxycortisol, (d)-11-deoxycortisol, (e)-androstenedione, (f)-testosterone, (g)-17α-hydroxyprogesterone, (h)-dehydroepiandresterone, (m)-progesterone).

In the control group (N=8), a positive correlation was found for the concentrations of progesterone (p=0.001; r=0.95), DHEA (p=0.027; r=0.77) and 17OHP (p=0.031; r=0.75) (Figure 5).

The overall correlations for the two groups are presented in Table 3.

Discussion

Unbound steroids are transferred from blood to saliva by passive diffusion along a concentration gradient and should reflect the level of free steroids in the blood serum from the total level of circulating steroids [18]. Saliva collection does not require highly trained personnel and is safe to work with and is easy to collect and store. The potential use of saliva for steroid hormone testing is very convenient for infants, children, and the elderly, as well as in circumstances where blood and urine sampling is not possible. Commercially available containers, such as Salivette®, are among the most widely used for saliva collection, although these containers are only officially approved for cortisol analysis and have been shown to be ineffective for some other steroid

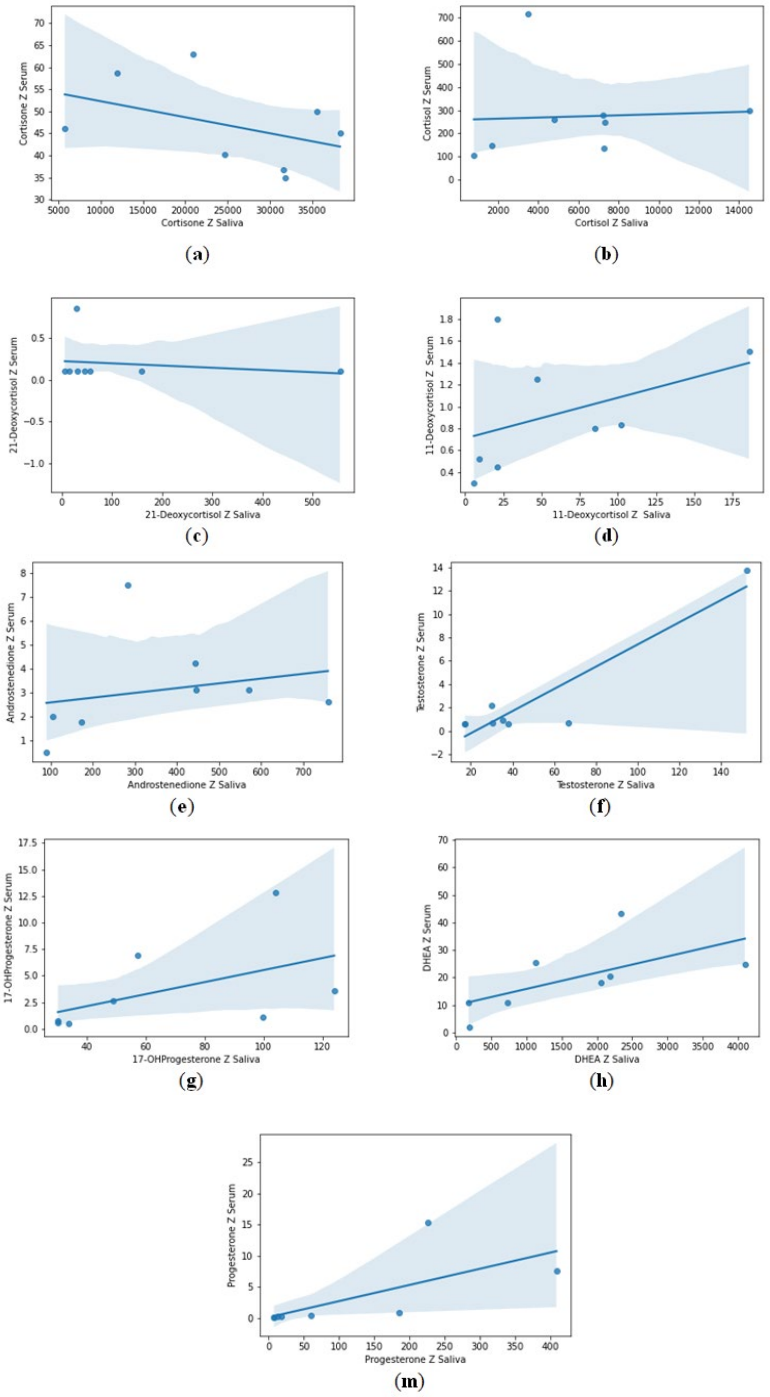

Figure 5: Correlation of steroids between blood and saliva in the control group ((a)-cortisone, (b)-cortisol, (c)-21-deoxycortisol, (d)-11-deoxycortisol, (e)-androstenedione, (f)-testosterone, (g)-17α-hydroxyprogesterone, (h)-dehydroepiandresterone, (m)-progesterone).

Table 3: Correlation of steroids between blood and saliva.

Criteria 1	Criteria 2	N	p, Spearman method	r
Cortisol Saliva	Cortisol Serum	16	0.594	-
Cortisone Saliva	Cortisone Serum	16	0.008	0.63
21-Deoxycortisol Saliva	21-Deoxycortisol Serum	16	0.001	0.74
11-Deoxycortisol Saliva	11-Deoxycortisol Serum	16	0.124	-
Androstenedione Saliva	Androstenedione Serum	16	<0.001	0.94
Testosterone Saliva	Testosterone Serum	16	0.261	-
17-OHProgesterone Saliva	17-OHProgesterone Serum	16	<0.001	0.85
DHEA Saliva	DHEA Serum	16	0.023	0.57
Progesterone Saliva	Progesterone Serum	16	0.025	0.56
Cortisol Z Saliva	Cortisol Z Serum	8	0.385	-
Cortisone Z Saliva	Cortisone Z Serum	8	0.32	-
21-Deoxycortisol Z Saliva	21-Deoxycortisol Z Serum	8	0.555	-
11-Deoxycortisol Z Saliva	11-Deoxycortisol Z Serum	8	0.183	-
Androstenedione Z Saliva	Androstenedione Z Serum	8	0.192	-
Testosterone Z Saliva	Testosterone Z Serum	8	0.233	-
17-OHProgesterone Z Saliva	17-OHProgesterone Z Serum	8	0.031	0.75
DHEA Z Saliva	DHEA Z Serum	8	0.027	0.77
Progesterone Z Saliva	Progesterone Z Serum	8	<0.001	0.95

hormones [15]. As the saliva collection system itself seems to be the most convenient for the analysis of the salivary steroid profile, so we decided to investigate other materials used as swabs. However, our results showed that the use of sorbent materials for saliva collection has a detrimental effect on salivary steroid analysis.

Apparently, this occurs because the analytes are sorbed to the surface of the sorbent material. We observed a pattern in which the degree of analyte loss increases with increasing lipophilisity of the steroid in the series Cortisone – Cortisol – 21-Deoxycortisol – 11-Deoxycortisol – Androstenedione – Testosterone – 17α-hydroxyprogesterone – DHEA – Progesterone.

Due to the lipophilic nature of steroids, it was assumed that the use of other highly absorbent materials, such as viscose and cellulose, as sorbent materials could eliminate the problems encountered when using the standard swabs provided in the Salivette® kits, as their structure appears to be more hydrophilic. Cotton swabs also showed an overestimation of the results for DHEA and Progesterone at low concentrations, which is due to the presence of substances in cotton that are analytically indistinguishable from these analytes. However, the results obtained indicate that the use of these materials is unacceptable for an adequate assessment of the steroid profile. Despite the results obtained, the saliva collection format using an absorbent material seems to be the most convenient, and further study of other absorbent materials seems quite interesting.

Currently, direct collection of saliva in polypropylene tubes is the most appropriate method for assessing the salivary steroid profile. However, there are some limitations to be aware of when collecting saliva: direct sample collection can be performed with or without stimulation of salivary secretion (e.g., citric acid, sugar-free gum, paraffin). Another important requirement is the absence of blood in the saliva due to mechanical damage in the oral cavity [15].

For further study, saliva was collected directly into polypropylene tubes without additional stimulation and we confirmed the correlation of steroids between the blood and saliva of patients with CAH, which has been previously demonstrated by other authors [16-19]. The weak correlation found for testosterone, androstenedione, and 11-deoxycortisol is apparently, seems to be due to an insufficient number of patients. However, increasing the number of patients will significantly improve the accuracy of reference intervals determination for salivary steroids. In this case, the sample collection and pretreatment of should be carried out by "passive drool" method in a polypropylene tube.

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