

Hormones in Saliva: Mode of Entry and Consequent Implications for Clinical Interpretation

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Assay of hormones in saliva would be more convenient than assay in blood, but there is no information on the route by which hormones enter saliva, information that would provide insight into the clinical value of such assays. We have examined the mode of entry of various hormones into saliva. The results suggest that unconjugated steroids enter saliva by diffusing through the cells of the salivary glands and that their concentration in saliva does not depend on the rate of saliva production. Conjugated steroids enter saliva via "ultrafiltration" through the tight junctions between the acinar cells, and their concentration in saliva is highly flow-rate dependent. Thyroxin and choriogonadotropin enter saliva via the ultrafiltration route or by contamination of the saliva by plasma or gingival fluid. We conclude that the salivary concentration of unconjugated steroids may usefully reflect the concentration of free (nonprotein-bound) steroids in plasma. Conversely, the concentration of conjugated steroids, thyroxin, and protein hormones such as choriogonadotropin in saliva probably does not reflect their concentration in plasma in any clinically useful way.

Additional Keyphrases: *steroids · protein hormones · thyroid hormones · thyroxin · choriogonadotropin · gingival fluid*

The clinical usefulness of measuring steroids in saliva has received much recent attention (1), and it appears that such measurements apparently can provide a useful index of unbound steroids in plasma in the case of many biologically important steroids. However, attempts to measure some steroid conjugates in saliva (1, 2) have revealed "anomalously" low concentrations. Similarly, thyroid hormones and some protein hormones such as choriogonadotropin are also present in saliva, and a knowledge of their concentration may also be clinically useful.

Many recent studies have been concerned with the relationship between concentrations of various steroid hormones in saliva and plasma (1), but there has been little discussion of the route by which steroids (or other hormones) enter saliva. We have examined the relation between saliva flow rate and salivary steroid concentration and between concentrations in serum and saliva of steroid hormones, thyroid hormones, and choriogonadotropin. We believe the resulting insight into the mode of entry of steroids and other hormones into saliva has important implications for the clinical usefulness of measuring these compounds in saliva.

Materials and Methods

Collection of Sample

While chewing unflavored chewing gum, subjects collected whole saliva directly into a small plastic vial (3). Such specimens were obtained from normal men and women except that those used for the studies of estriol and chorio-

gonadotropin were from healthy pregnant women, all of whom subsequently delivered normal infants. Gingival fluid was collected from subjects with mild gingivitis by drying the gums with a tissue and applying a disposable micropipette directly to the junction between tooth and gum. Gingival fluid was drawn into the micropipette by capillary action. Parotid saliva was obtained by placing a modified Carlson-Crittenden cup (4) over the opening of Stensen's duct. Pure parotid saliva was collected at either low flow rate (resting conditions; flow rate less than 60 $\mu\text{L}/\text{min}$ from the single parotid gland) or high flow rate (salivation stimulated by placing citric acid crystals on the tongue; flow rate greater than 350 $\mu\text{L}/\text{min}$ from the single gland). The dead volume of the Carlson-Crittenden cup and the tubing leading to the collecting tubes was kept to a minimum (<100 μL) to diminish problems of mixing of high- and low-flow-rate saliva.

Blood was collected by venipuncture from an antecubital vein into siliconized tubes. The separated serum was stored frozen at -20°C until assayed.

Procedures

Cortisol in serum and saliva was assayed by radioimmunoassay (RIA) as previously described (5). Dehydroepiandrosterone sulfate (DHEAS) in serum or saliva was determined by a direct RIA involving tritiated DHEA and an antibody raised against the hapten DHEA-3-hemisuccinate-human serum albumin. The antibody bound the DHEA and DHEAS equally (i.e., 100% cross reaction) but cross reacted negligibly or not at all with other steroids likely to be present in serum or saliva. We used charcoal coated with methyl cellulose to separate bound and free DHEAS, and determined the bound fraction by liquid scintillation spectrometry.

Unconjugated estriol in serum and saliva was determined by RIA as previously described (3).

The nonprotein-bound fraction of both cortisol and unconjugated estriol was determined by centrifugal ultrafiltration (3, 5-7) and the absolute concentration of free hormone in serum was then obtained by inference from the total serum concentration.

Conjugated estriol in serum and saliva was measured by first hydrolyzing the conjugates enzymically and then measuring the unconjugated estriol by RIA as previously described (3).

Human choriogonadotropin was measured by RIA with the use of an antiserum that bound equally the whole molecule of choriogonadotropin and its beta subunit, but showed only a 10% cross reaction with human lutropin (luteinizing hormone). The assay included a second-antibody precipitation step to separate free and bound hormone, and the standard used was the International Reference Preparation 75/537.

Thyroxin (T₄) in serum was measured by RIA with use of an antibody raised in sheep against a T₄-bovine serum albumin conjugate. 8-Anilino-1-naphthalene sulfonic acid was added to block binding of T₄ to endogenous proteins, and dextran-coated charcoal was used to separate bound and

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free tracer. In a direct assay of T4 in saliva we used 8-anilino-1-naphthalene sulfonic acid to block binding to endogenous proteins, and a similar separation with charcoal. The T4 concentration in extracts of saliva was also measured, which achieved a higher effective sensitivity by allowing a larger volume of saliva to be assayed. For this assay we extracted T4 from saliva by lyophilizing the saliva, dissolving the residue in 50 μ L of 25 mmol/L NaOH, adding 500 μ L of ethanol, mixing, and centrifuging, then removing the supernatant fluid and drying it. Samples of the dried supernate were then dissolved in buffer for assay. The concentration of free T4 in serum was measured with an Amerlex kit (Amersham, Buckinghamshire, U.K.). The T4 binding capacity of saliva was estimated by incubating T4 standard and tracer in 1 mL of saliva and then separating the bound and free tracer by use of charcoal.

The RIAs used were those in routine use in our hormone assay service; they meet the usual tests of validity for RIA, such as intra- and inter-assay precision, accuracy, and specificity.

For correlations, the line of best fit was determined by unweighted robust regression (8-10) and all tests of significance were based on nonparametric ranking methods (11).

Results

Figure 1 illustrates the relation between saliva flow rate and the concentration of cortisol and DHEAS in parotid saliva. The measured DHEAS concentration was markedly lower at the high flow rate than at the low flow rate, whereas the cortisol concentration was not significantly different at the two flow rates. The antiserum used for the DHEAS assay cross reacted equally with DHEA and its sulfate, DHEAS, and the concentration reported is the sum of DHEA and DHEAS. Thus the observed decline in DHEAS concentration with increased saliva flow rate was probably even greater than Figure 1 implies, because the DHEA contribution (probably about 1 nmol/L) to the total observed (DHEA plus DHEAS) would probably have remained constant at the two flow rates.

From an examination of the concentration of DHEA (12, 13) and DHEAS in serum and saliva (Table 1), it is evident that the DHEAS concentration in pure parotid saliva (about

3 nmol/L) is approximately 0.05% of the total concentration in plasma and is only approximately 1% as great as the unbound DHEAS concentration in plasma. Furthermore, the DHEAS concentration in whole saliva collected with the aid of unflavored chewing gum (to augment flow) averaged 16 nmol/L and was highly variable (0.6-70 nmol/L). Some normal subjects exhibited an apparent DHEAS concentration in whole saliva of 50 nmol/L for one sample and 10 nmol/L for a sample collected 1 h later. Moreover, the value for the first sample collected by a subject in the morning was often more than twice their daily mean value. These results are quite inconsistent with the relatively constant concentrations of plasma DHEAS (a consequence of its long biological half-life).

In an otherwise normal subject with mild gingivitis, "uncontaminated saliva" was collected from the floor of the mouth with a pipette, and saliva contaminated with gingival fluid was collected from around the teeth with a micropipette. Both samples were assayed for DHEAS. The DHEAS concentration in the "uncontaminated saliva" was 1.9 nmol/L and that in the "contaminated saliva" was 12.0 nmol/L.

The concentrations of conjugated and unconjugated estriol in serum and saliva (and the unbound unconjugated fraction in serum) were measured in time-matched samples of serum and saliva from women in the second half of normal pregnancy. The results (Table 2) show that the salivary unconjugated estriol concentration and the concen-

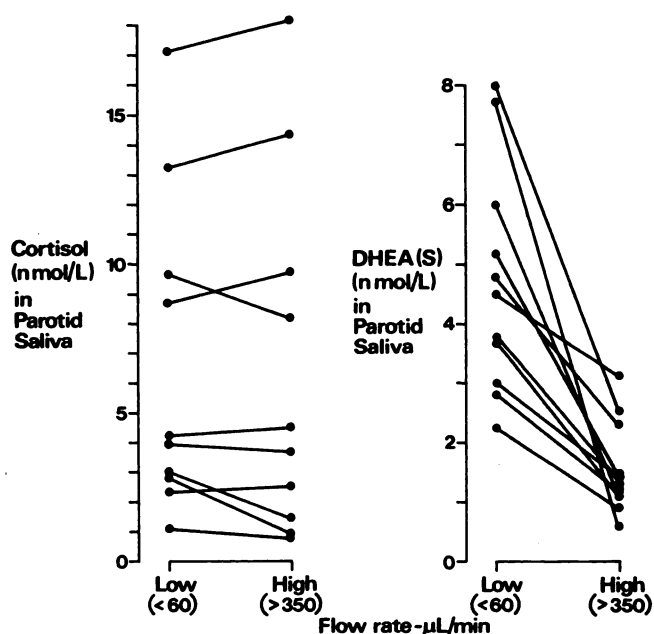


Fig. 1. Concentrations of cortisol and DHEAS in human parotid saliva at low and high saliva flow rates

Table 1. DHEA and DHEAS in Serum and Saliva

	Concn, nmol/L
<i>Serum</i>	
DHEA	
Unbound only	~2 ^a
Protein-bound + unbound	~24 ^a
DHEAS	
Unbound only	~250 ^b
Protein-bound + unbound (n = 50)	6000 (1500) ^c
<i>Saliva</i>	
DHEA + DHEAS	
Whole saliva ^d	16 (14) ^c
(n = 182)	Range 0.6-70
Parotid saliva	3.1 (2.2) ^c
(n = 23)	Range 0.5-8

^a Data from Dunn et al. (12) and Feher and Feher (13).

^b Derivation explained in text.

^c SD given in parentheses.

^d Whole saliva collected with the aid of unflavored chewing gum as a salivation stimulus to increase saliva flow rate.

Table 2. Estriol and Its Conjugates in Serum and Saliva during Normal Pregnancy (10 Mothers)

Weeks of gestation	Estriol concn, nmol/L				
	Saliva		Serum		
	Unconj. ^a	Conj.	Unbound ^a	Bound	Conj.
20	0.3	<1	0.2	2.7	20
19	0.6	<2	0.7	6.5	89
32	1.9	<3	1.8	18.7	255
36	2.1	<1	2.1	17.6	355
32	2.8	4	2.5	21.2	595
28	3.5	3	3.3	27.1	500
40	4.2	<1	3.9	31.2	496
40	5.6	3	4.6	40.9	601
33	5.8	10	2.1	18.0	480
34	5.9	3	4.4	43.2	502

^a Note the close correlation between the concentrations of saliva unconjugated estriol and serum unconjugated unbound estriol.

tration of unbound unconjugated estriol in serum have similar absolute values and are well correlated.

The concentration of unbound conjugated estriol in serum should be approximately 8% of the total conjugated estriol concentration in serum (12). In contrast, the concentration of conjugated estriol in saliva (Table 2) is less than 1% of the value for total estriol in serum; thus, the concentration of unbound conjugated estriol in saliva is less than one-eighth that of serum. Moreover, the concentrations of nonprotein-bound conjugated estriol in serum and saliva correlate poorly.

For concurrently collected specimens of serum and saliva from women during normal pregnancy the concentration of choriogonadotropin correlated significantly ($p < 0.005$) with that in serum (Figure 2) but the relationship (with $R^2 = 0.32$) has little predictive value. The mean choriogonadotropin concentration in saliva was only 0.4% that of serum.

Measurement of T4 in saliva as an index of free T4 in plasma was complicated by the presence of T4-binding protein(s) in saliva. We assessed T4-binding characteristics of saliva by measuring (a) B_0/T , the proportion of T4 tracer bound in the absence of added T4 standard, and (b) 50% displacement, the amount of T4 standard required to displace half of the bound tracer.

In a study in which five individuals collected a saliva sample every day for five consecutive days, the T4 binding was highly variable both between individuals (from 21% to 46% B_0/T) and for the one individual on different days (21% to 37% B_0/T on consecutive days for one individual). Similarly, the 50% displacement value for these saliva samples ranged from 0.5 to 8 nmol/L of T4.

Binding of T4 in saliva was blocked by 8-anilino-1-naphthalene sulfonic acid, 60 $\mu\text{mol/L}$, thus presenting the possibility of a direct assay of T4 in saliva. However, the resulting assay sensitivity was only 1 nmol/L, which was not adequate. With the extraction assay, the best sensitivity achievable was approximately 200 pmol/L, but most saliva samples had T4 concentrations less than this. In the routine assays performed as part of our clinical assay service the normal range for serum total T4 was 60–150 nmol/L and the normal range for free T4 (i.e., T4 unbound by serum proteins) was 11–22 pmol/L.

Discussion

Saliva is *not* formed by passive ultrafiltration but rather by an active energy-consuming process (14, 15) in which

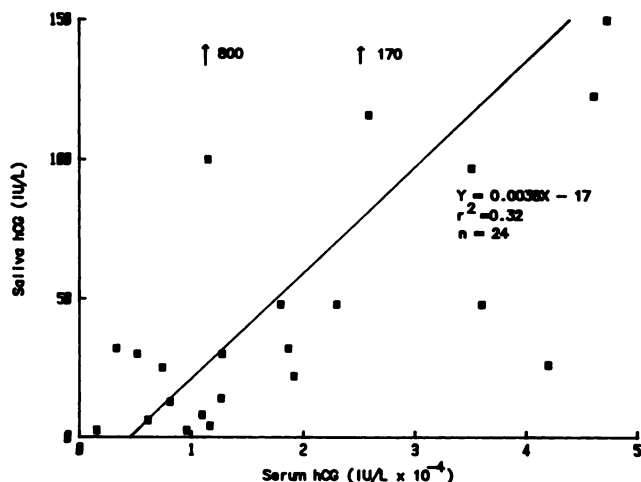


Fig. 2. Choriogonadotropin (hCG) concentrations (int. units/L) in time-matched samples of serum and saliva from women in normal pregnancy

sodium is pumped into the endpieces of the salivary gland, thus creating a difference in osmotic pressure between blood and saliva; consequently, water flows through the tight junctions (between the acinar cells) into the saliva. Thus the primary secretion (as it leaves the endpiece) is thought to be approximately isotonic with plasma. As the saliva moves down the ductal system of the salivary gland, the cells lining the ducts pump the sodium back into the blood; however, because there is very little transfer of water across the ductal membranes, the resulting saliva is hypotonic. The limited capacity of the ductal cells to pump the sodium out of saliva means that the sodium ion concentration in the secreted saliva is flow-rate dependent.

Thus, there appear to be two possible mechanisms by which plasma components may enter the saliva (apart from active transport):

- Intracellular diffusion; i.e., the compounds may diffuse into the acinar cells by virtue of their solubility in the lipid-rich cell membranes and then diffuse out the other side into the saliva. They may also pass by the same mechanism through the cells lining the ducts of the gland. This mechanism would be possible only for the lipid-soluble unconjugated steroids. The conjugated steroids (especially the sulfates) are not sufficiently lipid soluble. Similarly, thyroxin has only limited lipid solubility and choriogonadotropin is lipid-insoluble.

- Ultrafiltration. Along with the water that is to form the saliva, the plasma protein-unbound fraction of plasma may pass between the acinar cells (i.e., via the tight junctions).

Small polar molecules such as glycerol and sucrose are insoluble in lipids. Thus, if they are to appear in saliva, it must be via the ultrafiltration route. Under normal conditions glycerol (M_r 92) has a saliva/plasma ratio of 0.4 (15, 16) and thus must pass relatively freely from plasma to saliva, whereas sucrose (M_r 342) has a saliva/plasma ratio of 0.01 and accordingly must be largely excluded from saliva. Thus it would appear that for compounds entering the saliva via the tight junctions (the ultrafiltration route) there is a relative molecular mass cutoff of approximately 100–300.

Thus if steroids (average M_r approximately 300) could enter the saliva only via the ultrafiltration route, their salivary concentration would be less than 1% of the unbound concentration in plasma.

The salivary concentrations of the lipid-soluble, unconjugated steroids such as estriol (Table 2) (3), cortisol (5), and testosterone (17) closely approximate the unbound plasma concentrations. However, the salivary concentrations of the lipid-insoluble, conjugated steroids—DHEAS and estriol sulfate—are approximately 1% of the unbound plasma concentrations.

These data are consistent with the conclusion that the principal route of entry into saliva for the lipid-insoluble, conjugated steroids is via the tight junctions between the acinar cells (the ultrafiltration route), whereas the lipid-soluble, unconjugated steroids may also enter by diffusion through the acinar cells (the intracellular route).

The DHEAS concentration in parotid saliva was only 0.05% of the total plasma concentration, and the concentration in whole saliva was higher and more variable than that in parotid saliva alone. Similarly, the concentration in gingival fluid was higher than in whole saliva. Thus it seems likely that the increased, variable concentrations in whole saliva were a consequence of contamination of the saliva with traces of plasma (from minor abrasions to the gums or mouth) or gingival fluid (compared with the salivary gland, the gingival membrane is very "leaky" and becomes even more so in subjects suffering from gingivitis). Clearly, contamination of saliva with plasma or gingival

fluid is a major potential problem for measurement of salivary DHEAS, a compound for which the plasma/saliva ratio is 2000, but is unlikely to cause problems with estriol or cortisol, for which the plasma/saliva ratio is only 10.

The marked flow-rate dependence of salivary DHEAS concentration is consistent with the hypothesis that the DHEAS enters the saliva primarily by diffusion through the tight junctions between the acinar cells (the "ultrafiltration route"). In contrast, most of the cortisol in saliva appears to enter intracellularly via diffusion through the cells of the salivary glands; moreover, the diffusion rate for cortisol is clearly high enough to maintain a concentration equilibrium between the unbound fraction in plasma and the saliva fraction, independent of saliva flow rate.

In our experiments investigating steroid concentration at various saliva flow rates, we used a relatively normal stimulus to salivation (citric acid), and the DHEAS concentration showed a marked decrease with increasing saliva flow rate. However, the induction of sustained, very high saliva flow rates by pharmacological agents such as pilocarpine may produce a marked increase in salivary DHEAS concentration. Under these conditions the saliva/plasma ratio for sucrose may increase 20-fold (16, 18) and the tight junctions between the acinar cells are thought to enlarge, allowing passage of compounds of M_r up to 1000. This may further complicate the interpretation of salivary DHEAS concentrations.

The saliva flow rate/salivary concentration relationships for many other steroids of biological interest (e.g., testosterone, dihydrotestosterone, androstenedione, estradiol, progesterone, etc.) are difficult to delineate in normal individuals because their low salivary concentration precludes their precise measurement in the small volumes of saliva available at low saliva flow rates (though the relationship for progesterone and the estrogens should be definable during pregnancy, when their concentrations are much higher). However, a prediction of the concentration vs flow-rate relationship can be made for various steroids, on the basis of information on their diffusion rates through biological membranes, coupled with our knowledge of the relationship for cortisol. Thus Scheuplein et al. (19) showed that, for diffusion through hydrated stratum corneum, cortisol had a far lower diffusion rate than the other commonly measured unconjugated steroids such as progesterone, testosterone, and estradiol. Thus, if the salivary and plasma concentrations of unbound cortisol are at equilibrium even at high flow rates, then so should be the concentrations of the other, unconjugated, more rapidly diffusing steroids, and thus the salivary concentrations of these latter analytes should not show a dependence on saliva flow rate.

Many plasma-derived proteins of clinical interest (e.g., choriogonadotropin, pituitary hormones, albumin, etc.) are present in saliva in trace amounts. The choriogonadotropin concentration in whole saliva does show a correlation with gestation period (Figure 2), but the relationship has little predictive value. The plasma/saliva ratio for proteins such as choriogonadotropin and albumin is of the order of 10 000/1, and the origin of such molecules in saliva is probably primarily due to contamination with plasma or gingival fluid. Accordingly, the presence or absence of some proteins in saliva may provide qualitative results, e.g., measurement of choriogonadotropin for a pregnancy test, but attempts to interpret variations in the salivary concentration of these proteins are not likely to be of value.

In plasma of normal subjects the ratio of total T4 to free T4 is approximately 5000/1 with a free T4 concentration of about 15 pmol/L. We were not able to assess the T4 concentration in saliva accurately because of limited assay

sensitivity. However, our results suggest that, although most individuals have saliva T4 concentrations less than 200 pmol/L, some samples may exceed this value. The protein binding of T4 in whole saliva is probably due to trace contamination of the saliva with plasma: we found that a 5000-fold dilution of plasma in assay buffer would bind a similar amount of T4 tracer.

Because of the very large total-to-free ratio for T4 in plasma, contamination of saliva with plasma or gingival fluid is likely to cause a large variability in the concentration of T4 in saliva. Correspondingly, we found that the T4 binding capacity of saliva varied markedly both between individuals and within individuals on consecutive days. Accordingly, measurements of the T4 concentrations in whole saliva are unlikely to give an accurate index of the plasma concentrations of free T4.

In summary, the following conclusions may be drawn from our results:

- The lipid-soluble unconjugated steroids (such as cortisol, estriol, testosterone, progesterone, etc.) enter saliva predominantly via the intracellular route; their salivary concentrations are *not* dependent on saliva flow rate, and their salivary concentrations closely approximate their unbound concentration in plasma. Accordingly, the salivary concentration of these hormones may provide a useful clinical index of their unbound concentrations in plasma.

- The conjugated steroids, which are essentially lipid insoluble (e.g., DHEAS and conjugated estrogens), are largely excluded from saliva, although small amounts do enter via the "tight junctions"; their salivary concentrations are dependent on saliva flow rate, and their salivary concentrations are so small as to be readily affected by trace contamination by gingival fluid or plasma. Accordingly, their salivary concentration appears unlikely to provide a useful clinical indication of their plasma concentration.

- Similarly, the concentration in whole saliva of any compound, such as choriogonadotropin or T4, for which the plasma/saliva ratio is more than 1000/1, is unlikely to be clinically useful because of the problem of trace contamination by plasma or gingival fluid.

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