

## Current Status of Salivary Hormone Analysis

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**BACKGROUND:** Saliva, which offers a noninvasive and stress-free alternative to plasma and serum, is a widely accepted sample source for analysis of steroids and also of certain amines and peptides. In recent years, numerous publications have described the use of salivary hormone analysis in many fields of clinical and basic research.

**CONTENT:** This review provides an overview of the current applications of salivary hormone analysis. A description of the different modes of hormone entry into saliva is followed by a detailed description of analytical methods and approaches for reliable collection of saliva, including several interesting applications in diverse fields including psychiatry, stress research, clinical endocrinology, sports medicine, and veterinary medicine.

**SUMMARY:** Although saliva has not yet become a mainstream sample source for hormone analysis, it has proven to be reliable and, in some cases, even superior to other body fluids. Nevertheless much effort will be required for this approach to receive acceptance over the long term, especially by clinicians. Such effort includes the development of specific and standardized analytical tools, the establishment of defined reference intervals, and implementation of round-robin trials. One major problem, the lack of compliance sometimes seen in outpatient saliva donors, requires strict standardization of both collection and analysis methods to achieve better comparability and assessment of published salivary hormone data.

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The use of saliva as a sample source for hormone analysis has become increasingly attractive for clinicians and researchers because collection of saliva offers a noninvasive and stress-free alternative to collection of plasma and serum, the traditional matrices for the de-

termination of endocrine parameters such as steroids, amines, and peptides.

For 40 years, endocrinologists have used saliva as a supplementary sample matrix. Prior reviews, from Riad-Fahmy et al. in 1982 (1) to Lewis in 2006 (2), have focused on salivary analysis of many steroids, although numerous reported studies have demonstrated that saliva monitoring is a useful alternative method for analyzing hormones of other biochemical origins.

This review provides an overview of well-established procedures and promising future methods of salivary hormone analysis and the application of these methods in fields as diverse as psychology, clinical endocrinology, fertility, sports medicine, and behavioral research.

### Transfer of Hormones to Saliva

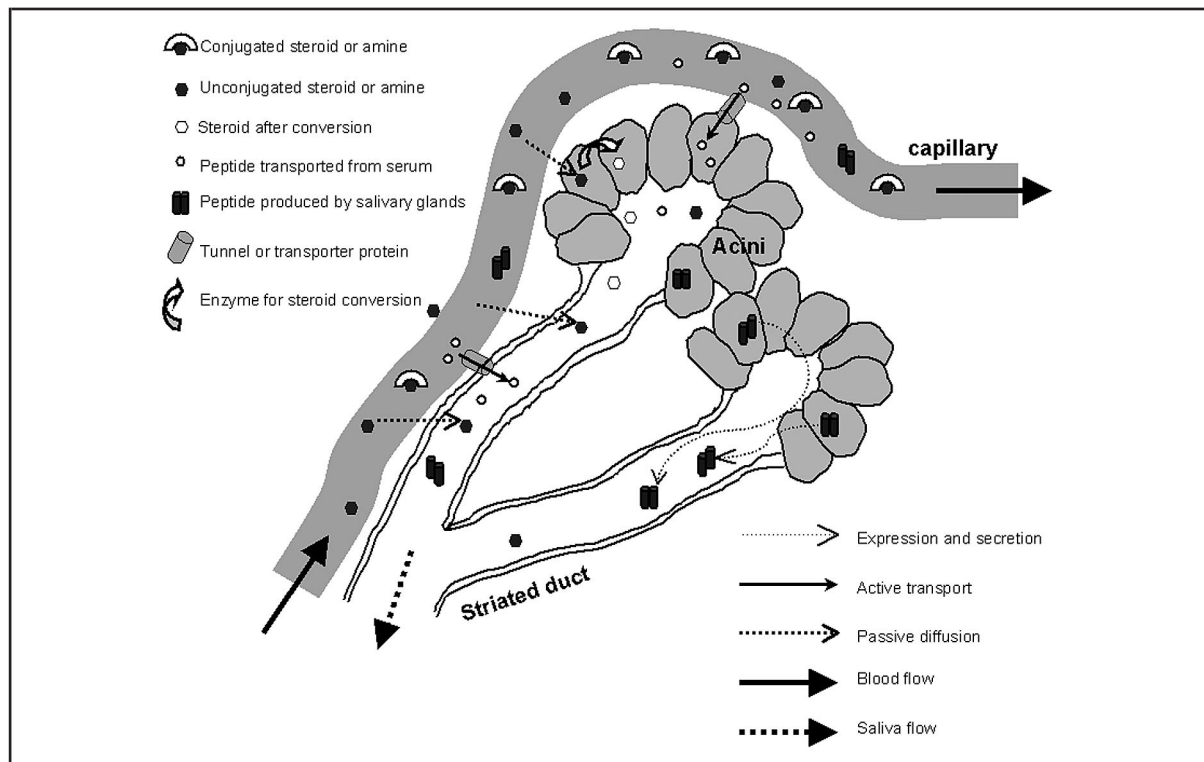
Saliva has multiple functions, including moistening the mucosa of the upper digestive tract, which makes saliva indispensable for swallowing food. Enzymes that aid in digestion, such as amylase, are present in saliva. Saliva also contributes to the immune response, containing secretory IgA and antibacterial peptides that inhibit oral microbial flora. Moreover, saliva is a carrier of signal molecules, which are either transported into the salivary glands from the blood vessels or are independently produced by the glands (Fig. 1).

The speed at which hormones can be transferred from blood into saliva is controlled by passage through the lipophilic layers of the capillaries and glandular epithelial cells. Consequently, lipophilic molecules, such as steroids, are transferred through these barriers more rapidly than hydrophilic molecules, such as peptides.

Lipid-soluble steroids (1) and amines (3) travel by passive diffusion, passing through the capillary wall, basement membrane, and acinar cells of the secretory endpieces along a concentration gradient. Thus, salivary concentrations of lipid-soluble, unconjugated steroids such as cortisol reflect the approximately 10% unbound plasma concentrations, whereas lipid-insoluble, conjugated steroids such as dehydroepiandrosterone-sulfate make up less than 1% of the unbound plasma concentration (4).

Steroids are usually not metabolized to more polar, water-soluble metabolites by the salivary glands, in

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**Fig. 1. Transport mechanisms of hormones into saliva.**

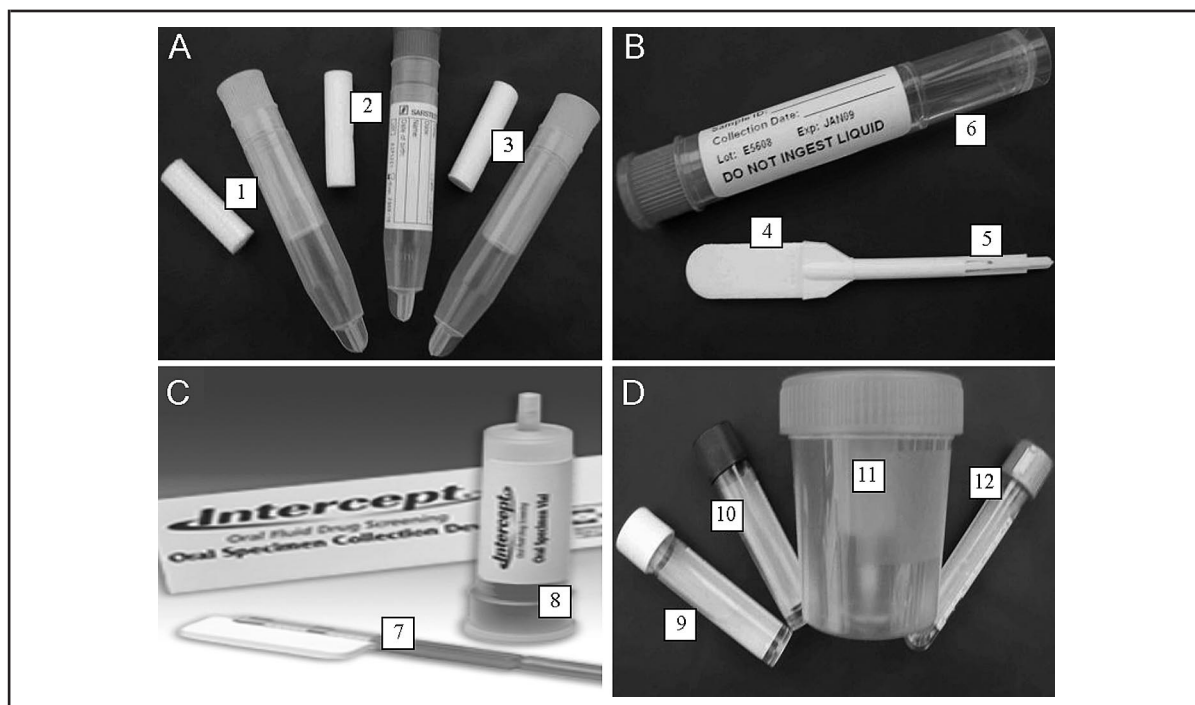
Steroid hormones, being conjugated to specific transport proteins in the circulation, enter the glands by passive diffusion. In specific cases, an enzymatic conversion occurs (e.g., cortisol to cortisone). Peptides enter the salivary glands either by active transport mechanisms (e.g., insulin) or are expressed and secreted by the salivary glands themselves (e.g., epidermal growth factor).

contrast to substances excreted by the kidneys. Saliva is therefore preferable to urine as a noninvasive alternative for steroid determinations. Significant conversion of cortisol to cortisone in the salivary glands by  $11\beta$ -hydroxysteroid dehydrogenase II, however, causes a shift from cortisol to the inactive ketoform (5). This enzymatic conversion is often underappreciated when salivary cortisol is measured and explains certain discrepancies in the cortisol concentrations reported in the literature. Depending on the cross-reactivity of the antibodies used in the immunoassays, the data often reflect not only cortisol concentrations but falsely increased measurements of both glucocorticoids together.

Some peptide hormones such as insulin (6) are actively transported into saliva from their tissues of origin, whereas others, including certain cytokines (7, 8), are produced by the salivary glands themselves. Specific transporters for insulin are present in the oral mucosa, as proven after sublingual application in rats (9). The selectivity of this active transport is demonstrated by the fact that insulin appears in saliva at similar con-

centrations to those in plasma after glucose uptake, but the cleavage product C-peptide, which is of a similar size to insulin, does not enter the saliva.

Peptides of salivary glandular origin are secreted by exocytosis directly into the acinar lumen. An active, energy-dependant mode of transport causes difficulties in interpreting the salivary concentrations, because concentrations of salivary peptides do not always correlate as well with plasma concentrations as do concentrations of passively diffused steroids. For certain cytokines, such as tumor necrosis factor and leptin, lower concentrations appear in saliva than in plasma (10, 11). For other cytokines, such as epidermal growth factor (12) and ghrelin (13), saliva concentrations have been observed to be equal to or higher than those in plasma. Because reference intervals are not available for salivary concentrations of peptide hormones, researchers generally use salivary peptide hormones qualitatively as marker substances for specific oral diseases rather than as a noninvasive alternative to plasma for quantitative measurements.



**Fig. 2.** Different commercial collection devices for saliva.

(A), Three versions of the Salivette® with inlays of polyester (1), polyethylene (2), or cotton (3) and the accompanying collection tubes. (B), The Quantisal® consists of a cellulose pad (4) on a plastic stem with a flag window (5), which signals adequate volume by a color shift. A sample container with preservative buffer (6) is part of the system. (C), The Intercept® also consists of a cellulose pad (7) on a plastic stem, with the sample being transported in a centrifuge container without preservatives (8). (D), The SCS® consists of a mouth-rinsing solution (9), yellow collection solution (10), a collection container for draining the saliva mix (11), and a storage unit coated with preservative powder (12).

## Collection and Analysis of Saliva

### TECHNIQUES FOR THE COLLECTION OF SALIVA

Although the selective collection of gland-specific saliva by either suction or cannulation of the ducts is possible, mixed saliva is the only practical collection method for routine, open-field studies and outpatient sampling.

In many studies, participants have been asked to spit or drool directly into the collection-tube. Active spitting is considered sufficient stimulus, producing up to 1 mL/min, whereas passive drooling is considered to provide nonstimulated saliva. This direct collection has several disadvantages, one of which is a substantial social barrier to spitting (14), especially in geriatric patients. Moreover, the presence of xerostomia aggravates collection by passive drooling, particularly in older patients (15). To increase salivary flow, the application of citric acid to the tongue has commonly been used to induce maximal secretions of 5–10 mL/min, but the use of citric acid has been reported to interfere with immunoassay analysis by decreasing the pH of the

sample (16). As a consequence, in most studies saliva is collected with absorbent tissues in the mouth and then extracted by centrifugation.

### COMMERCIAL COLLECTION DEVICES

Several commercial collection devices have been established as reasonably suitable for collection of saliva samples for salivary hormone analysis. Because the collection procedure should not affect salivary concentrations, any absorption or modification of the analytes must be avoided. Certain materials, such as Parafilm® (17) and cotton (18), are known to absorb target molecules from the saliva, leading to falsely decreased measurement results.

Currently the most commonly used systems are the Salivette® (Sarstedt, Fig. 2A), the Quantisal® (Immunoanalysis, Fig. 2B), and the Intercept® (Orasure Technologies, Fig. 2C).

All these systems use a collection pad that is inserted into the mouth, either under the tongue or in the cheek. The absorbent pad is kept in the mouth to soak up saliva for a standardized period of time (1–2 min

preferred), and then the pad is transferred to a storage container. Centrifugation is used to remove the saliva sample for use.

These devices have been shown to generate reproducible results for the analysis of many salivary steroids and peptide hormones (19). The cotton version of the Salivette, however, should definitely be excluded because of interferences in the assays.

In contrast to the above-mentioned devices, the Saliva Collection System® (Greiner BioOne, Fig. 2D) uses a very elaborate procedure involving rinsing and collection liquids. The analytical results achieved with this device have been reliable for most peptide hormones and steroids (19).

#### ANALYTICAL METHODS FOR THE MEASUREMENT OF HORMONES IN SALIVA

*Preanalytical precautions.* Reliable salivary analysis requires collection, storage, and preparation of the specimen, and protocols for these tasks may vary depending on the analyte of interest.

Steroids, owing to their high stability in saliva, present the fewest problems. Even in samples stored at room temperature glucocorticoids and androgens are stable for a few days in untreated saliva, but storage for longer than a few days under these conditions should be avoided (20). Adding preservatives significantly prolongs the stability of salivary steroids (21) so that samples from outpatients can be sent to a laboratory by mail (22).

Measurement of salivary peptides or amines, such as insulin (6) and melatonin (23), requires greater attention to sample collection and handling. Peptides are more likely than steroids to adsorb to the surface of collection tubes, and such adsorption may cause significant loss of sample peptides. Moreover, saliva contains proteolytic enzymes that rapidly degrade peptide hormones (24), particularly hormones with a short half-life, such as ghrelin (13). Adsorption loss and degradation can be minimized by the use of appropriate collection devices, low-protein-binding cryotubes for storage, and added preservatives such as EDTA.

*Immunological methods.* Immunoassays have been widely used for analyzing salivary hormones because they are relatively simple to use, require small sample volumes ( $\leq 100 \mu\text{L}$ ), and are analytically sensitive. However, immunoassays do not always have the analytical specificity required for distinguishing the desired hormones in the presence of cross-reactants commonly seen in neonates, pregnant women, and patients with certain diseases.

The first immunoassays developed for the analysis of steroid hormones in saliva were “home-made”

methods, based either on in-house antibodies and tracers (25, 26) or adaptations of commercial RIAs for the saliva matrix (27). In most cases commercial RIA adaptation included an adjustment of the protein content of the standard buffer and the dilution of the samples, leading to better precision and accuracy of salivary hormone measurement. These adjusted RIA methods, however, were applied only for steroid but not for peptide analysis.

RIAs have now been completely replaced by nonradioactive ELISA methods. Several assay-manufacturing companies (<http://www.salimetrics.com/>; <http://www.drg-diagnostics.de/>; <http://www.dslabs.com>) offer FDA-cleared assays for salivary steroids. Salivary assays for peptide hormones and growth factors are not yet available from these vendors.

The development of assays for salivary peptide hormones and growth factors is important because these components of saliva appear to be the most promising for use as biomarkers of disease. For example, salivary assays for peptides and proteins useful to the clinically oriented researcher will soon become available for use in head and neck cancer (28) and oral cancers (29, 30). Innovative new multiplex immunological methods, based on analytical chip systems (31) or microbead assays, are future techniques likely to be used for rapid multianalyte determinations of salivary peptide hormones.

*Chromatographic methods.* Modern liquid chromatography, coupled with mass spectrometric (MS) detection, has provided vast improvements in analytical specificity and sensitivity. These methods, based on the generation of gaseous ions of the molecule or specific fragments, characterized and quantified by the specific mass-charge ( $m/z$ ) ratio, have been used for 2 different applications.

One application used in saliva samples is the detection of new peptides or proteins that can be identified by either MALDI-TOF MS (32) or SELDI-TOF MS (33). This approach has been used to investigate the saliva proteome encompassing the numerous salivary peptide hormones and growth factors, but quantification of the detected hormones has not been the main emphasis of these studies.

A second application has been the use of liquid chromatography–tandem MS for quantification of salivary steroid hormones (34, 35) and small peptides and amines with molecular weights below 5 kDa, such as ghrelin (36) and melatonin (37). It appears very likely that in future many of the problems described for immunoassays will be overcome by use of this very specific and sensitive method, which enables screening of a complete profile of steroids in one sample.



### Current and Future Applications of Salivary Hormone Analysis

We next consider the potential applications of salivary hormone analysis for clinical and basic research use. Some of these analyses are already accepted tools for diagnostic and therapeutic evaluations, whereas others are still being developed and provide promise for the future.

#### NONINVASIVENESS AS A PREMISE IN PSYCHOLOGY AND STRESS RESEARCH

Cortisol, one of the substances most frequently investigated in saliva, is a hormone that acts by increasing the rate of gluconeogenesis during stress. Side effects of sample collection must be avoided in studies investigating the effect of stressful events or moods, such as depression. Thus, the noninvasive, stress-free collection of saliva has become particularly popular for use in psychological investigations (38). In the wide variety of studies on stress and endocrinology, there is a consensus that salivary cortisol increases dramatically under chronic stress, as shown in victims facing life-threatening situations (39), in socially isolated or disadvantaged people (40), and in patients suffering from depression (41). Other studies, however, have not substantiated the relationship between stressful situations and increased salivary cortisol concentrations (42).

The unexpected negative outcome of some studies may have a physiological or pathological basis. Such results are more likely attributable to methodological aspects, however, because strict standardization of collection and comparison to established reference intervals appear to be more problematic in psychological investigations than those carried out in other fields. The collection time, in particular, is very influential in the assessment of salivary cortisol, owing to the circadian rhythm of adrenal secretion. In addition the effects of stressful events can cause important variations in the concentrations of salivary cortisol. Stress studies are often based on the evaluation of the correlation of self-assessed stress surveys with salivary stress hormone concentrations, and in these studies compliance of the study participants in following the collection protocol was found to be a major problem affecting data validity. In a salivary cortisol sampling protocol, self-reported compliance of collection time was shown to substantially overestimated actual compliance, especially in the absence of objective monitoring by a supervisor (43).

The pituitary-adrenal cortex axis is responsible for the chronic stress response, but acute stress parameters such as catecholamines are difficult to assess in saliva because of the low concentrations and rapid degrada-

tion of epinephrine and norepinephrine (44) and the difficulty of stabilizing these hormones in the sample.

Evidence exists, however, that other substances cosecreted with catecholamines can serve as an alternative index of adrenergic activity and can be reliably measurable in saliva owing to their greater stability. In particular, chromogranin A (CgA), an acidic peptide stored by the adrenal medulla and coreleased with catecholamines, has become more accepted in studies of the endocrine acute stress response. Salivary CgA was found to increase rapidly during prolonged noise (45) and to decrease significantly in individuals being tested in a setting with humorous content (46). Accordingly, CgA in saliva could be a valuable parameter for measuring acute stress response under controlled study conditions. Another classical surrogate for adrenal medulla activity is  $\alpha$ -amylase, which, although not a hormone, shows the same excretion patterns as catecholamines (47). Because assays for amylase are more easily available in smaller clinical laboratories, saliva analysis of this enzyme may offer an interesting alternative for adrenergic activity testing.

#### SALIVARY CORTISOL AS A PROVEN PARAMETER IN THE DIAGNOSIS OF CUSHING SYNDROME

By far the best established and accepted application for hormone analysis in saliva is the use of salivary cortisol in the diagnosis of Cushing Syndrome, as highlighted in a recent review (48). The disease is caused by sustained pathologic hypercortisolism due to excessive corticotropin secretion by pituitary gland tumors, by ectopic corticotropin secretion, or by corticotropin-independent cortisol secretion by the adrenals.

One of the hallmarks of Cushing syndrome of any origin is the abrogation of circadian rhythmicity in adrenal cortical secretion. In contrast to healthy individuals, who show a decrease in cortisol levels from high morning to low evening values (49), patients with Cushing syndrome do not display any reduced cortisol secretion even in late evening (50). Consequently, the measurement of increased late evening or midnight cortisol is considered a very simple and useful way to screen for Cushing syndrome (51). The diagnosis of endogenous hypercortisolism can be made without the disturbing experience of stressful hospitalization, by obtaining a saliva sample from patients at bedtime, even at home under accustomed conditions.

In 1985, Luthold et al. described the use of late-night salivary cortisol to detect Cushing syndrome (52). In later studies, this method was confirmed to yield excellent overall diagnostic accuracy for Cushing syndrome (53–55).

Because of the varied clinical manifestations of Cushing syndrome, however, late-evening salivary cortisol alone has limitations and is not intended to re-

place current standard screening tests, but rather to supplement the analysis of 24-h urinary cortisol (56). Although the disease is much rarer in children than in adults, the noninvasive and convenient outpatient collection of saliva is especially advantageous for pediatric patients (57, 58).

In addition to screening for Cushing disease of any type, the salivary cortisol test can be extremely useful in the evaluation of patients suspected of having intermittent Cushing syndrome (59). Owing to the convenience of sample collection, the patient can provide samples several evenings in a row. Because of the simplicity of multisample collection, saliva is also the preferred sample for use in monitoring Cushing patients undergoing dexamethasone suppression test (60), for which there is no overlap between values obtained in patients and in healthy controls (61). Under standardized conditions, a remarkable reproducibility was observed for night-time cortisol during overnight dexamethasone suppression (62). Consequently, these short-term interval (15 min or less) sample-collection protocols can provide useful information for the individual differential diagnosis of Cushing disease (63).

#### SALIVARY STEROIDS IN THERAPEUTIC CONTROL OF CONGENITAL ADRENAL HYPERPLASIA

With most children being afraid of venipuncture, saliva collection has proven very beneficial in pediatric medicine, although saliva has not completely replaced other sample sources for the full spectrum of analyses required for diagnosis or therapy of endocrine disorders. The most common use of saliva is to obtain additional information on the performance of glucocorticoid replacement therapy in patients with congenital adrenal hyperplasia.

In congenital adrenal hyperplasia, a genetically derived enzymatic block limits the adrenal synthesis of both mineralo- and glucocorticoids. In the most common version, caused by 21-hydroxylase deficiency, a precursor steroid of cortisol, 17 $\alpha$ -hydroxyprogesterone (17OHP), is present in much higher concentrations in the circulation and also in saliva of affected than in healthy individuals. The accumulated 17OHP can be reliably measured in saliva samples collected at intervals during the day, usually in the morning, at noon, and in the evening (64). This saliva profile is used along with urinary steroid excretion to assess the quality of substitution therapy, providing information on the efficacy of suppressive regimes in the treatment of congenital adrenal hyperplasia, which uses cortisol, cortisone-acetate, prednisolone, and dexamethasone as glucocorticoid replacement (64). Congenital adrenal hyperplasia also involves increased adrenal secretion of androgens, leading to virilization of female patients. Therefore in addition to 17OHP, salivary

androstendione, a direct marker of adrenal androgen secretion, has become an accepted marker for the effectiveness of suppression therapy, although its use in monitoring therapy for this disease is less widespread (65).

The benefit of saliva use for outpatient monitoring is that saliva profiles can be collected at home and sent to the laboratory between regular medical examinations, providing information on the therapeutic status between yearly visits without the need for the patient to report to the clinic.

In addition to the typical 3-sample profile, more frequent saliva collection has been shown to yield valuable information on the kinetics of orally administered glucocorticoids (66) and to aid in the adjustment of individual substitution doses to obtain optimized suppression of 17OHP. As already discussed in the section on psychological applications, however, the predominant problem is not the reliability of salivary 17OHP analysis but the limited compliance of children and adolescents. Sloppiness in the collection procedure, such as sampling after instead of shortly before taking the substitution pill, and inadequate storage or labeling of the samples can obstruct the successful estimation of the therapeutic quality.

#### SALIVARY SEX STEROIDS IN FERTILITY RESEARCH

Sex steroids, including androgens and estrogens, and also gestagens, have been analyzed successfully in saliva for years. In assessing the ovarian cycle, saliva samples have been demonstrated to enable differentiation between the follicular and luteal phase for both estradiol (67) and progesterone (68). Saliva collected daily throughout the menstrual cycle shows a specific pattern, with a midcycle rise and a peak in the early luteal phase. Mean salivary progesterone concentrations in the follicular phase, as has been described in several publications, range from 20 to 100 pmol/L, whereas peak concentrations during the periovulatory period may attain 300 pmol/L. This significant difference is consistently reported and clearly allows for an assessment of ovarian function. Salivary progesterone and, more rarely, estradiol are still assessed in conjunction with urinary steroid profiling.

A major focus of testosterone analysis in saliva is in fertility research for the treatment of male hypogonadism. Testosterone measurement in saliva has been successfully used to differentiate between eugonadic and hypogonadic men, demonstrating a very high correlation with free testosterone levels in serum. This association understandable, because specific sex-steroid binding globulins play only a minor role in saliva. Because of the close correlation of salivary testosterone to serum free testosterone, salivary testosterone concentration was established as a biomarker in the diagnosis

of male androgen deficiency (69), with a cutoff value for salivary testosterone of 0.2 nmol/L, above which hypogonadism can be ruled out. Reliable analysis of salivary sex steroids is very challenging, however, and Granger and colleagues stress the limitations of this method (70). Salivary testosterone measurements can be substantially influenced by sample collection methods and are sensitive to storage conditions. Moreover, the variability of androgen levels in relation to age, especially during puberty, makes a clear interpretation of salivary testosterone values difficult, particularly because of the scarcity of defined age- and sex-dependent reference intervals (70).

An unfortunate development in salivary hormone determination during the last few years must be mentioned in this section. An increasing number of vendors offer saliva analysis directly to patients through the internet. Customers order a collection set and send the saliva sample back to the vendor, who provide the results of the (hopefully valid) steroid analysis by mail. When presenting these data to their physician, patients are surprised to learn that the results are practically worthless for a clinical interpretation. If these data were to be used to initiate hormonal replacement therapy without consulting an experienced and critical endocrinologist, the procedure would carry incalculable risks for the customers' health. Physicians should therefore inform their patients of the lack of reliability of these nonapproved services.

#### SALIVARY HORMONES IN SPORTS MEDICINE

The assessment of hormonal changes during athletic activity can provide information regarding training success that may be valuable to the scientific staff backing athletes and trainers. The collection of blood and urine samples before and after training is therefore routine. Obtaining these bodily fluids to assess changes during training is problematic, however, because an interruption in the training regimen is necessary. Saliva samples are easily accessible during training, as demonstrated by several reports of analysis of androgens and glucocorticoids during soccer (71), rugby (72), or judo competitions (73).

Unfortunately, physiological hormonal changes are not the only focus of saliva analysis in sport medicine. Another area of investigation is doping, the use of performance-enhancing drugs. Some of these drugs are hormones, such as anabolic steroids and certain cytokines, such as erythropoietin.

Androgens, synthetic or natural, are still important as prohibited stimulatory substances. Athletes use them to gain more muscle mass and to reduce pain during extreme physical exertion. As already mentioned, androgens can be reliably assayed in saliva, as these steroids pass the endothelial-epithelial barriers by

passive diffusion. Long-term use of anabolic steroids leads to an altered androgen profile. Therefore, one criterion for suspected doping with the androgen testosterone is the quotient between testosterone and epitestosterone, assessed in urine. Normally this quotient is approximately 1. If this quotient shifts towards testosterone (much more testosterone is detected than epitestosterone), the athlete is suspected of taking illegal androgens. Because both androgens can be measured in saliva, it would seem beneficial to take saliva samples during training or competition conditions. Saliva samples are easier to collect in the presence of witnesses than are urine samples, which are collected while the sample donor is behind a screen.

Synthetic androgens such as tetrahydrogestrinone pass the epithelia in the same way as endogenous androgens, and again saliva allows rapid access to the sample for screening purposes without violating the privacy of the athlete, as has successfully been shown for nonhormonal doping drugs. At the time of this report, the official pages of the World Anti-Doping Agency offered no information as to whether programs exist that use saliva samples for the detection and control of doping.

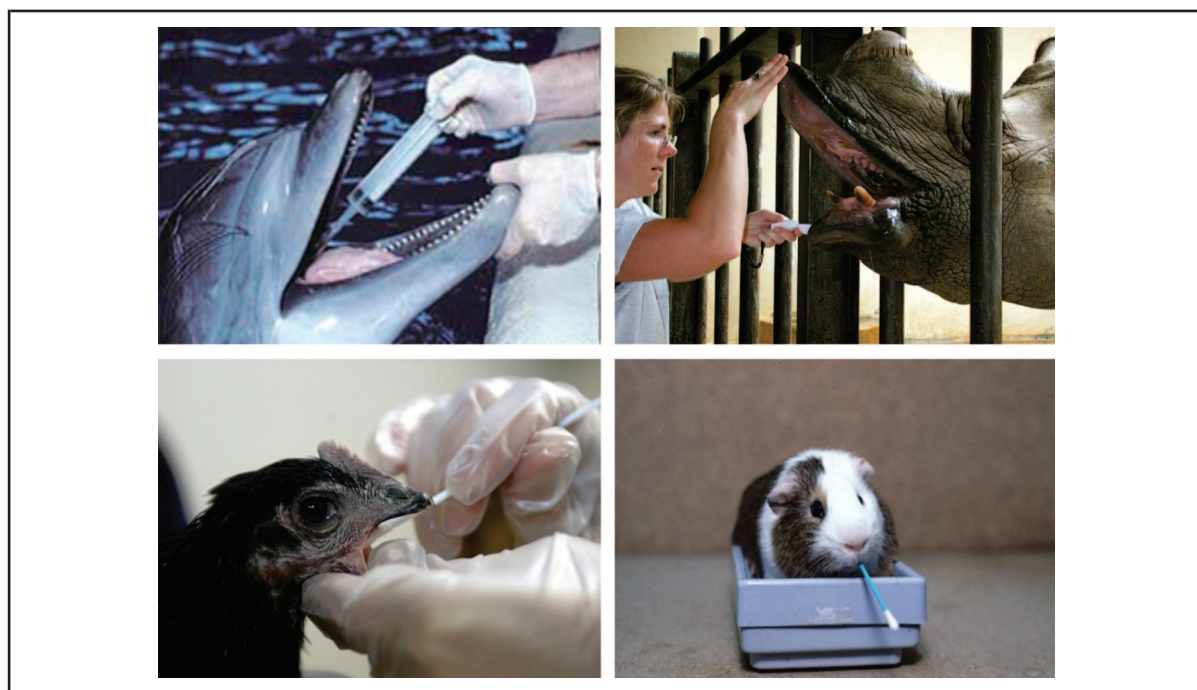
Exogenous peptides used in doping include human growth hormone to enhance muscle growth and erythropoietin to increase the number of erythrocytes. Sporadic reported investigations have demonstrated the presence of these 2 peptides in saliva (74, 75) at very low concentrations (% of plasma concentrations).

Because the salivary glands do not express these peptides, transport from the blood vessels through the epithelia into the salivary system is likely. If endogenous peptides can pass through the epithelia, then recombinant human growth hormone or erythropoietin may also be transferred the same way. Recombinant peptide hormones that have been produced by molecular biological methods sometimes carry a tag that is fused with the peptide for purification reasons. To date, no reported data have demonstrated results in saliva indicating the use of these cytokines as doping substances, such as human growth hormone or erythropoietin values higher than physiological reference intervals or the presence of tagged peptides in saliva.

Salivary hormone analysis is a promising method for sports medicine and doping control, but much work is needed before the use of saliva samples in this arena receives the acceptance attained in other medical disciplines.

#### SALIVARY STEROID ANALYSIS IN VETERINARY MEDICINE AND BEHAVIORAL RESEARCH

Endocrinological use of saliva samples in veterinary medicine is frequently associated with fertility research and the breeding of valuable or endangered animals.



**Fig. 3.** Examples of saliva collection in trained animals: Dolphin (Brookfield Zoo, Chicago), rhino at fertility testing (Smithsonian National Zoo, Front Royal, VA, courtesy of Janine L. Brown), chicken and guinea pigs in research institutes (Department of Animal Physiology, University Bayreuth, Germany).

For example, Brown et al. found salivary steroid analyses to be reliable in assessing the ovarian cycles of the Indian rhinoceros (76). By studying the normal cycle length of the female rhinoceros, investigators detected pregnancy through analysis of salivary  $20\alpha$ -hydroxypreg-4-en-3-one in captive African black rhinos (77). Obtaining saliva from rhinos is possible only from animals accustomed to the keepers in the park or zoological garden, but according to the authors, saliva was particularly useful for situations in which it was difficult to collect uncontaminated urine and feces (76).

Saliva has also been used to assess gluco- and mineralocorticoids in animals. As in humans saliva collection in animals is presumably less stressful than blood collection, as confirmed by interesting studies in such diverse mammals as guinea pigs (78), marmosets (79), and even dolphins (80) (Fig. 3).

According to the authors of these studies, salivary measurement allows assessment of hormonal changes in behavioral research without the effects of blood-collection stress, providing valuable information on the endocrine status of animals in dominance hierarchies or during gestation. Although stress-free and standardized sample collection may be easy and advantageous in humans and trained laboratory animals, saliva col-

lection appears to be extremely difficult and challenging in captive zoo animals.

#### SALIVARY MELATONIN AS A TOOL IN CHRONOBIOLOGY

Melatonin is an amine produced in the pineal gland during darkness. The melatonin signal is part of the system that regulates the circadian cycle. In the normal biological rhythm, high melatonin values occur during the night. During the day, the concentrations fall to very low basal values (81). Because melatonin passes the epithelial barriers in the same way as steroids, it is more frequently analyzed in saliva than in plasma.

In today's business world people travel rapidly between different time zones. The inner clock often cannot adapt to this shift, causing the phenomenon of jet-lag, attributable to misalignment between circadian rhythm and destination local time. A number of studies have described the use of salivary melatonin in chronobiological research. The military and the National Aeronautics and Space Administration are especially interested in assessing the hormonal status of flight crews before and during long-distance flights (82, 83) to investigate method for adapting the sleep-waking phases to mission requirements. In these circumstances, noninvasive saliva collection is obviously preferable to venipuncture blood collection.



Civilian areas of research have focused on the adaptation of humans to changes in the day-night cycle or to different qualities of light exposure. In both psychiatry and industrial medicine, salivary hormone analysis may be important for studying the circadian rhythms in shift workers exposed to artificial light over long periods of time (84).

### Summary and Conclusions

This review has provided an overview of the current progress in salivary hormone analysis, highlighting applications in research and therapy. Although still not a very popular subject, the assessment of hormones in saliva is receiving increasing interest.

For specific investigations, particularly in psychiatry, stress research, and pharmacokinetics, saliva analysis may deliver equivalent or even better results than blood analysis. The predominant advantage of salivary hormone analysis is the noninvasiveness of collection procedures, enabling samples to be obtained from patients afraid of venipuncture, especially children and phobic patients, without an unwanted adrenal stress response. A disturbing influence of stress-induced adrenal activity is less likely in saliva samples, making salivary glucocorticoid values, for example, very reliable compared to serum values in stress research, pediatric applications, and the diagnosis of Cushing syndrome.

The convenience of rapid short-interval sampling and the availability of non-protein-bound hormones are the other main advantages of salivary analysis. Daily collection during the menstrual cycle, multiple sample collections per day to observe circadian rhythms, and even frequent collections at shorter intervals for kinetic studies are possible and, in trained individuals, can be easily performed at home. Saliva analysis also makes samples accessible under conditions in which blood donation is impossible, such as during sport training.

Major applications for salivary hormone analysis in sports medicine may develop in the future.

Several factors have limited the acceptance of salivary analysis even after years of investigation. Much effort will be required in the long term to achieve broader acceptance of saliva use by clinicians. Specific and standardized analytical tools are required, as have been in place for decades in the analysis of serum and plasma. The definition of reference intervals related to age, sex, and time of day and the establishment of round-robin trials for salivary hormone measurements are overdue. No data are available to indicate whether commercial immunoassays from different manufacturers deliver equivalent results, especially for well-established parameters like cortisol, testosterone, and melatonin.

Another critical problem for study coordinators and physicians is the lack of compliance with established collection procedures sometimes seen in outpatient saliva donors. To overcome this problem, strict protocols for collection procedures and patient training are mandatory.

In summary, standardization of both collection and analysis must be achieved to gain better comparability of published salivary hormone data and to improve the acceptance of saliva as a reliable additional sample matrix in endocrinological investigations.

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