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Innovative Non- or Minimally-Invasive Technologies for Monitoring Health and Nutritional Status in Mothers and Young Children

Human Saliva as a Diagnostic Specimen[1]

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ABSTRACT Human saliva can be easily obtained by noninvasive techniques and contains many analytes of interest for screening, diagnosis and monitoring. These include steroid and other nonpeptide hormones, therapeutic drugs, drugs of abuse and antibodies. Numerous studies in the past 40 y have shown correlations between serum and saliva levels. Both diurnal and monthly profiles of hormone levels parallel traditional serum patterns. Multiple specimens for steroid hormone analysis can be easily collected by the patient, at home, to monitor fertility cycles, menopausal fluctuations, stress and other diurnal variations. Drug doses can be monitored without inconvenient and costly visits to blood-drawing facilities. Antibody levels can be determined to screen for infectious diseases. Saliva can be collected directly by spitting into a tube or with one of several devices, each of which has its own special advantages and disadvantages. Salivary levels of steroid hormones and other analytes that are protein bound in serum reflect the unbound and active concentration of the hormone. Saliva can be used as a diagnostic specimen not only to obtain information more inexpensively and efficiently than serum, but also to provide information not readily available from serum testing. J. Nutr. 131: 1621S–1625S, 2001.

KEY WORDS: • *saliva* • *hormone assay* • *diagnostic specimen* • *drug testing* • *noninvasive techniques*

Saliva is a readily available specimen, which can be collected by noninvasive procedures and contains many hormones, drugs and antibodies of interest in screening and diagnosis (Brandtzaeg 1989, Major et al. 1991, Read 1989, Knott 1989). With a salivary specimen, one can collect multiple specimens from the same individual at the optimum times for diagnostic information. This is of particular value for steroid hormones because many have diurnal or monthly variations. Saliva can be collected in remote sites by unskilled personnel and, with certain collection devices, is stable at ambient temperatures for several weeks (Schramm and Smith 1991, Wade and Haegle 1991, Frerichs et al. 1992, George and Fitchen 1997).

Advantages and disadvantages

Saliva as a diagnostic medium has many advantages over serum for a large variety of types of testing.

- Because saliva can be collected without breaking the skin or entering the body in any other way, it has obvious advantages for multiple noninvasive collections and for obtaining samples from those whom, for cultural reasons or age or because of physical or mental handicaps, it would be unethical to collect blood samples.
- The free, rather than the protein-bound, hormone is considered to be the active component in blood (Read 1989). The steroid hormones in saliva are thought to reflect the free hormone concentration. Therefore, saliva levels are a more accurate reflection of the active hormone in the body, especially for steroid hormones, which are strongly bound in blood by specific binding globulins (Read 1989).
- Saliva can be collected with devices so that it will be stable at room temperature for extended periods. One approach is to filter out bacteria and enzymes. The saliva is collected by osmosis into a membrane sack that only allows small molecules to enter (Schramm and Smith 1991). Another approach is to add preservative to the saliva. Saliva samples are collected for human immunodeficiency virus (HIV)[3] antibody by absorption onto filter paper and then the paper is put into a tube containing a small amount of buffer and preservative (Frerichs et al. 1992, George and Fitchen 1997).
- Many of the hazards associated with blood collection do

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[3] Abbreviations used: HIV, human immunodeficiency virus; SLPI, secretory leukocyte protease inhibitor; IgA, immunoglobulin A; DHEAS, dehydroepiandrosterone sulfate; IgG, immunoglobulin G; ODS, Oral Diffusion Sink; FDA, Food and Drug Administration; IgM, immunoglobulin M.

not apply to saliva. There is no need for sharps, which have the potential for cross contamination among patients when used improperly and present a danger to health care personnel. Because of the low concentrations of antigens in saliva, HIV and hepatitis infections are much less of a danger from saliva than from blood (Major et al. 1991).

- Because of diurnal and monthly variations, several steroid hormones need multiple samples collected early in the morning or late at night or at the same time every day for a month to give meaningful results (Read 1989). Such collections are often very expensive, inconvenient or impossible to do with blood.
- Nonpolar analytes are released into saliva through the membranes via a mechanism that is not flow dependent. Therefore, concentrations remain constant relative to blood levels with stimulated and unstimulated collections (Vining et al. 1983a).
- The presence of secretory leukocyte protease inhibitor (SLPI) may be another factor contributing to the safety of saliva as a diagnostic specimen. SLPI expresses antiviral activity against free HIV-1 in a model using monocyte- and lymphocyte-derived tumor cell lines (Hocini et al. 2000). Because SLPI does not interfere with trancytosis of cell-associated virus, this study suggests that the inhibitory effect is restricted to HIV-1 in corporal fluids, such as saliva, cervico-vaginal secretions or breast milk.

The problems associated with saliva are primarily caused by lack of familiarity with the medium and can be remedied by use of available information and experience.

- Most saliva collections for steroids are performed by direct spitting into a tube or absorption onto cotton balls. These samples are not sterile and are subject to bacterial degradation over time. However, most analytes are stable at ambient temperature for ~7 d so they can be collected and shipped without refrigerant. Absorbing specimens on cotton may contribute interfering substances to the extract. Salivary results for dehydroepiandrosterone, testosterone and progesterone were artificially high when collected on cotton, whereas results for secretory immunoglobulin A (IgA) were artificially low (Shirtcliff et al. 2001). The devices mentioned above that collect sterile samples have not been widely used and standardized for most saliva assays.
- Interpretation of saliva assays is still difficult. Although diurnal and monthly patterns generally parallel serum values, absolute ranges show variability in different studies. Few studies of normal individuals, controlling for known variables, such as pH, time of day and month and medications, have been performed using the recently developed high sensitivity enzyme immunoassays, such as those sold by Diagnostic Systems Laboratories (Webster, TX), Salimetrics (State College, PA) and American Laboratory Products Co. (ALPCO) (Windham, NH).
- Because blood concentrations of steroid hormones are several-fold higher than saliva levels, much has been written about the problems of contamination from bleeding gums. A urine dipstick shows a positive reaction for hemoglobin in saliva collected after a person has brushed his teeth, even if the saliva was negative before brushing. However, this problem has been much over stressed because the contamination measured here is <1:5000 and the saliva to serum ratios of steroid hormones are ~1:200. A specimen with a 1:5000 contamination with blood is pink. I have looked at thousands of saliva specimens collected from around the world and very few have been colored.
- Polar hormones, such as thyroxine, and the peptide hormones are subject to variation by flow rate, so reliable levels cannot be obtained in saliva at this time (Read 1989). However, antibody testing, when one is looking for a qualitative result as for HIV or hepatitis, can be performed with accuracy with a saliva specimen (Major et al. 1991, Frerichs et al. 1992, Granade et al. 1995).
- Proficiency-testing programs are not yet available for saliva. A few kits offer saliva controls with the reagents (Diagnostic Systems Laboratories, ALPCO, Salimetrics). A positive and negative control for HIV was developed for use in HIV testing in Third World countries (Hofman et al. 1993). This lack of proficiency controls makes validation of laboratory tests for certified laboratories difficult.

Entry of analyte into saliva

Saliva may be classified by the gland by which it is secreted. However, most saliva steroid measurements are made on whole saliva because the collection of parotid saliva or palatine secretions require the presence of a person to do the collection negating the advantages of saliva as a diagnostic medium and adding little to the value of the information obtained.

Most of the information on steroid hormones in saliva deals with unconjugated hormones. Vining et al. (1983a) report on detailed studies concerning cortisol, estriol and dehydroepiandrosterone sulfate (DHEAS). Their results suggest that unconjugated steroids enter saliva by diffusing through cells of the salivary glands and that their concentration does not depend on the rate of saliva production. In contrast, conjugated steroids, such as DHEAS, enter saliva via ultrafiltration through the tight junctions between acinar cells, and their concentration in saliva is dependent on flow rate of saliva. Although cortisol concentrations are constant in samples collected with and without stimulation, DHEAS concentrations drop from ~5 to 2 nmol/L.

There are several sources of antibodies in saliva (Brandtzaeg, 1989). At least 95% of the IgA in saliva is produced by the salivary gland immunocytes. Most of the IgA produced is in the dimeric form, secretory IgA, consisting of two IgA molecules connected by a J chain and secretory piece. However, most salivary immunoglobulin G (IgG) enters the oral cavity by passive diffusion primarily through the gingival crevices and is increased in subjects with periodontal disease.

Collection of saliva

How the saliva is collected is dictated by the analyte being tested and the information desired. Some general considerations apply. Aspects to be considered before beginning to collect saliva include the following:

- Whether resting or stimulated saliva will be collected, and, if stimulated, how will it be stimulated. Many studies have used sugar-free gum and found it not to interfere with steroid assays.
- The amount of saliva needed to complete the analysis.
- Pretreatment of saliva before assaying and storage until assay. This depends on the method of collection and whether a preservative is used.
- If the patient may be taking medications or have a disease causing dry mouth.

TABLE 1

Hormone levels: comparison of serum and saliva concentrations

Hormone	No. of specimens	*R* value	Author
17-OH progesterone (total serum vs. saliva)	13	0.98	Price et al. 1979
Estriol (unconjugated)	24	0.97	Vining et al. 1983a
Cortisol (free)	93	0.97	Vining et al. 1983b
Human chorionic gonadotropin	24	0.56	Vining et al. 1983c
Progesterone (total serum vs. saliva)	96	0.88	De Boever et al. 1986
Estradiol (total serum vs. saliva)	14	0.82	Worthman et al. 1990
Cortisol (total serum vs. saliva)	13	0.93	Diagnostic Systems Laboratories

- Metabolism of steroids in the salivary glands or the mouth.
- Whether quantitative or qualitative assays will be run on the specimen.

The most common way to collect saliva is by direct spitting into a tube. There are a couple of devices that filter the specimen, one by placing a small membrane sack in the mouth [Saliva Sac; Pacific Biometrics, Seattle, WA (Schramm and Smith 1991)] and the other with a tiny plastic tube containing cyclodextrin to bind the analyte [Oral Diffusion Sink (ODS); Saliva Testing and Reference Laboratory, Seattle, WA (Wade and Haegle 1991)]. Absorbent pads or balls are used to collect saliva, especially for qualitative testing (OraSure; Epitope, Beaverton, OR and Salivette; Sarstedt, Newton, NC). The absorbent pad is immersed in a small amount of fluid containing preservative to stabilize the specimen for several weeks. The ODS has the interesting property of being able to collect an average level over several hours. The device is suspended in the mouth by means of dental floss and saliva bathes it for the desired number of hours. The patient can sleep or do normal activities other than eating or drinking. The level obtained by extracting analyte from the device is the average over the time period (Wade and Haegle 1991).

Validation of saliva measurements

Saliva testing has been around for a long time. Many saliva/serum correlations have been performed. Although theoretically one would like all correlations to be performed with the free hormone because saliva concentration reflects the free level, this has not always been possible. Correlations with both free and total hormone appear in the literature.

In one study, plasma-free testosterone concentrations, determined by an equilibrium dialysis method, were compared with salivary testosterone concentrations in normal female subjects and in female patients with polycystic ovarian syndrome. The data were consistent with a linear regression of slope 1 and correlation coefficient of 0.9 (Riad-Fahmy et al. 1982). Another study reported in the same article compares free and salivary cortisol with a correlation coefficient of 0.97.

In children with congenital adrenal hyperplasia, 17-OH progesterone levels were measured in plasma and saliva (Price et al. 1979). The comparison was done between total plasma 17-OH progesterone and salivary 17-OH progesterone. The correlation coefficient (plasma/saliva) was 0.97. In this study, patients were followed with saliva and plasma levels taken at five times over the course of a 24-h period. The pattern was similar. It also showed a large variation in concentration at different times of day, emphasizing the ineffectiveness of procedures based on single sampling regimens to monitor poorly controlled patients. The routine stress-free collection of small aliquots of saliva at 1- to 2-h intervals ensures more effective replacement therapy.

A recent correlation was performed by Diagnostic Systems Laboratories showing the correlation between total blood cortisol and salivary cortisol using their enzyme-linked immunosorbent assay kit (Diagnostic Systems Laboratories). The correlation coefficient in this case was 0.93. The participants were probably young healthy employees with normal blood protein levels (personal communication, Durham, S).

A summary of some correlation studies is shown in **Table 1**. All of these show correlations of >0.8 except for human chorionic gonadotropin. As stated earlier, peptide hormone measurements in saliva are not reliable for a number of reasons having to do with mode of entry and susceptibility to degradation.

With the recognition of rising numbers of HIV infection in Third World countries in the early 1990s, a noninvasive screening method became of great interest. Correlations of saliva and serum assays were good. As an example, Major et al. (1991) looked at saliva from seropositive and negative individuals with a commercial (Cambridge Bioscience, Worcester, MA) and an in-house assay. The results are typical, showing 97–100% agreement (**Table 2**). In another study (Frerichs et al. 1992), duplicate vials of serum and saliva were collected from 879 high risk and 1039 low risk subjects from Myanmar. Using serum testing to define HIV status, the sensitivity for saliva testing was 90.5% and the specificity was 98.3%.

A later study (Granade et al. 1995) used a collection device for saliva that collected 1 ml of saliva on a thick filter paper pad (OmniSal; Saliva Diagnostic Systems, Vancouver, WA) from 149 known positive HIV individuals and 138 healthy individuals in Mexico. The sensitivity (compared with serum) for detection of HIV in saliva was 100% for the three enzyme immunoassays used and the specificity ranged from 90 to 98%.

In development (Orasure Technologies, Beaverton, OR) is a rapid test for HIV using saliva. The specimen is collected with a sampling pad between the subject's gum and cheek. The pad is then placed in a developer solution. Results of a lateral flow immunoassay can be read in <20 min (Clin. Lab. News 2001).

Research has demonstrated that saliva contains sufficient oral mucosal transudate, a serum-derived fluid that enters saliva through the gingival crevice and across oral mucosal surfaces to detect antibodies to various bacterial and viral diseases (George and Fitchen 1997). Sensitivity and specificity data for testing for HIV antibodies have been described above. A Food and Drug Administration (FDA)-approved test for HIV based on oral mucosal transudate is commercially available (Epitope). Saliva testing for measles has been reported for

TABLE 2

HIV 1 comparison: serum and saliva[1]

HIV status	Serum	Saliva assay 1	Saliva assay 2
Positive	103	101	103
Negative	74	76	74

[1] Data from Major et al. 1991.

both IgG and immunoglobulin M (IgM) antibodies (Brown et al. 1994). Measles-specific IgM was detected in 92% of adequate saliva samples (71/77) collected from patients with serum positive for measles IgM. An IgG antibody capture enzyme-linked immunoabsorbent assay was developed for detection of measles-specific IgG in oral fluid (Nigatu et al. 1999). The test was evaluated by comparing oral fluid and serum samples from 787 subjects in rural Ethiopia. By comparison with serum, oral fluid had a sensitivity of 97% (95.9–98.2) and a specificity of 90% (81.9–94.3). Despite these results, no salivary commercial assays are offered for any infectious diseases except HIV.

Drugs can also be measured in saliva. Some drugs, which have very little protein binding, have approximately the same level in saliva as serum. Others with more protein binding in serum behave more like steroid hormones. In one study (Riad-Fahmy et al. 1982) free plasma to total plasma and saliva to total plasma ratios for therapeutic drugs with varying degrees of protein binding were compared. The ratios were similar (**Table 3**). A saliva-screening test for drugs of abuse has been FDA-approved to measure tetrahydrocannabinol, cocaine, opiates, phencyclidine and amphetamines (Intercept; Epitope).

Cotinine levels are of interest to confirm the integrity of information received from questionnaires regarding smoking behavior. Both insurance companies and government nutrition programs offer benefits to nonsmokers over smokers, so they are particularly interested in an objective monitor. Willers et al. (2000) showed saliva and urinary cotinine to correlate with each other and to a lesser degree with plasma and with detailed questionnaires standardized to categorize smoking behavior. Prokhorov et al. (2000) refer to cotinine testing as the "gold standard for measuring nicotine intake."

Of perhaps more interest than saliva/serum correlations are correlations with physiological indices. Walker et al. (1979) measured progesterone in samples taken every day throughout a menstrual cycle for nine normal women and three infertile women. The normal women showed a typical sustained luteal rise and peak. None of the infertile women showed a sustained luteal rise.

An assay was developed to measure cortisol levels collected with the ODS device (Hofman 1995). This assay was used to compare saliva cortisol with average plasma levels collected from an indwelling catheter every 30 min. During the 4 h of greatest interest for cortisol levels, midnight to 4 AM, the participants collected saliva with the ODS device(Wade and Haegle 1991). **Table 4** shows the comparison of the single saliva value with the average of eight plasma values (Hofman, L. F., unpublished results). The correlation coefficient for the average plasma level and the saliva level obtained from the ODS collection was 0.96.

TABLE 3

Ratio of free to total drug concentration: drugs listed in order of increasing binding by plasma proteins[1]

Drug	Free/total in plasma	Saliva/plasma total
Antipyrine	0.9	1.0
Aminopurine	0.85	0.8
Digoxin	0.77	0.78
Phenytoin	0.10	0.09
Tolbutamide	0.09	0.012

[1] Data from Riad-Fahmy et al. 1982.

TABLE 4

Cortisol comparison of average plasma concentration versus ODS saliva concentration

ID	Average 4 h plasma nmol/L	ODS 4 h saliva nmol/L
Patient A	116	13
Patient B	348	32
Patient C	91	2.2
Patient D	397	50

[1] Plasma concentrations averaged over 4 h show a correlation with ODS values over the same 4 h of: $r = 0.96$; $y = 0.13x - 6.5$ (Hofman, L. F., unpublished data).

Cortisol profiles on normal subjects show the familiar diurnal variation, high in the morning and low at night (Read 1989). Although fibromyalgia and rheumatoid arthritis patients showed higher average salivary cortisols than did healthy controls, they maintained a normal diurnal variation (Catley et al. 2000). Melatonin shows the opposite profile, low in the morning and high at night (Hatonen et al. 1996, Voultsias et al. 1997). Cortisol in obesity is a much studied and complex problem (Bjorntorp and Rosmond 2000a). The study of the contribution of the hypothalmic-pituitary-adrenal axis has been facilitated by saliva cortisol collection (Bjorntorp and Rosmond 2000b). Diurnal measurements under undisturbed conditions can be made that follow discrete periodical elevations of cortisol secretion during everyday conditions. Testosterone profiles in men also show a diurnal variation that is much less prominent in women [**Fig. 1** (Hofman, L. F., unpublished results)].

Nighttime salivary cortisol measurements have been used as a simple noninvasive outpatient screening test of Cushing's syndrome in patients (Gafni et al. 2000). A midnight cortisol value of >7.5 nmol/L excluded all healthy children and identified 13 of 14 patients with Cushing's syndrome. The diag-

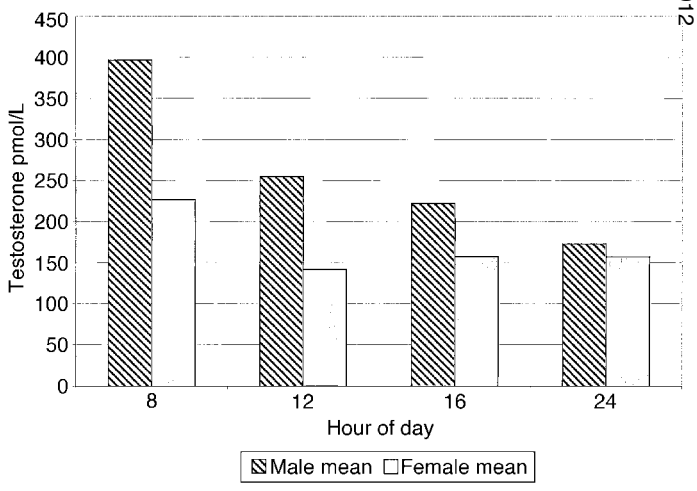

FIGURE 1 Testosterone: diurnal variation in normals. Six normal males and four normal females collected saliva samples at 8, 12, 16 and 24 h in a single 24-h period. Testosterone was assayed using an adaptation of an enzyme immunoassay from Diagnostic Systems Laboratories. The mean was determined separately for male and female values for each time. The average SD for the male values was 33% of the mean; for the female values, 28% of the mean (Hofman, L. F., unpublished results).

nostic accuracies of midnight salivary cortisol and urinary-free cortisol per square meter were the same (93%).

Salivary estriol has been shown to be an accurate way to predict preterm labor (Heine et al. 1999). Subjects collected unstimulated saliva between 9 AM and 8 PM at home and sent the samples to the clinic. Samples were tested for unconjugated estriol. A finding of levels > 2.1 ng/ml on two consecutive tests was considered a positive finding, predicting preterm delivery. Six hundred and one women, including women of high and low risk for preterm delivery, completed the study. There were 23 spontaneous preterm births and 578 term deliveries. The salivary estriol predicated the appropriate outcome 91% of the time.

Conclusion

Saliva collection allows the measurement of analyte levels in multiple samples collected at home or in remote locations. When properly instructed, most individuals can collect adequate specimens without professional help. Step-by-step instructions and tubes that do not leak are the main requirement for getting good specimens from motivated individuals. Specimens can be collected at room temperature and mailed to the laboratory without refrigerant. Children generally have lots of saliva and find spitting fun. The main problem holding back more general use of saliva is the lack of data on normal levels and effects of medications. The only salivary kits that are FDA approved at this time are those associated with the OraSure collection system for HIV and drugs of abuse (Epitope), although kits for steroid hormones (Diagnostic Systems Laboratories and Salimetrics) and secretory IgA and melatonin (ALPCO) are available for research use and may be submitted for FDA approval in the near future.

Studies of correlations between saliva and serum and/or urine have shown that saliva is an easily obtainable reliable diagnostic specimen for steroid and some other hormones and many drugs and antibodies. Of even more significance are studies that correlate saliva with symptoms and expected patterns of hormone variation. Saliva as a diagnostic specimen can give not only the same information as serum testing, but also additional or new information that cannot be obtained from serum.

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