Steroid Hormones in Serum and Saliva

Contents

2. Introduction

For almost a century various steroid hormone determinations have been performed from a variety of body fluids in the diagnostic laboratory to gather relevant information and assist clinicians in the diagnosis of various pathological conditions. Perhaps the most notable activity occurred in the 1960's when new analytical methods emerged that demonstrated superior analytical sensitivity at the low end of expected concentration ranges.
In the following discussion, we describe various analytical procedures for the determination of steroid hormones, which have a characteristic molecular structure as shown on the title page of this pamphlet. The structure consists of 3 hexagons and one pentagon linked together in classical biochemical configuration. Following is a list of the more prominent steroid hormones.

Cortisol	commonly associated with stress
Testosterone	male hormone responsible for sexual function
Estradiol	primary estrogene in men, children and non-pregnant women
Progesterone	primary gestagene, in women related to pregnancy
DHEA	primary androgene, metabolized in blood to DHEA-sulfate (DHEA-S)

The concentration of the biologically active fraction of these steroid hormones in circulation is extremely low. Therefore it is important to use sensitive laboratory tests in order to quantify these low levels of analyte. Use of less sensitive lab tests may result in inaccurate assessment which can, in turn, lead to mis-interpretation of patient condition. Unfortunately there are still analytical methods in routine laboratory use which lack appropriate analytical sensitivity. Following is a discussion of currently available analytical procedures used to measure steroid hormones in blood and saliva.

2.1. Blood, urine and saliva as analytical material

Today, steroid hormone testing is done primarily from blood products (serum or plasma). Serum is prepared from whole blood after clotting and centrifugation. Thus, serum does not include clotting factors. Plasma is prepared by collecting whole blood in a device containing anti-coagulants, which prevent clotting. After centrifugation, the supernatant (plasma) is separated. This plasma fraction contains clotting factors.

Use of urine samples to measure steroid hormones is no longer common. For a urine sample to be useful, a 24-hour urine should be collected and tested. This means the patients must collect their total urinary output over a 24 hour period.

Testing methodology of steroid hormones in saliva was first published in the 1980's and has since been a subject of debate in medical science. Saliva testing was not immediately popular in the clinical arena due to relatively large sample volume requirements, the need for extraction procedures in the testing labs, and the common use of radio-immune assays to perform the lab tests (radioactive lab reagents). These features are not attractive to a medical diagnostic laboratory. Only at the very end of the last century were new saliva testing procedures developed. The newer procedures require small sample volumes and non-radioactive test reagents. These methods are still not commonly found in diagnostic laboratories although they are gaining in popularity.

A discussion of the new methods for steroid hormone testing in saliva follows, beginning with a comparison of salivary and serum sampling processes. One significant advantage of saliva testing is that it is a non-invasive procedure, whereas a serum sample requires venipuncture. This is especially important when testing children, although many adults are uncomfortable during venipuncture sampling as well. In addition, saliva sampling can be done casually throughout the course of a day without the need for a clinic visit and phlebotomy (venipuncture) procedures. Multiple saliva samples can be collected easily within a short time span or over the course of a day, which is frequently desirable when assessing steroid hormones.

2.2. Expected Concentration Ranges

Due to the low concentration of steroid hormones in saliva, accurate lab testing methods need to achieve a detection limit in the low picogram range, if not less than 1 picogram. To better understand the meaning of this statement, and the potential importance of saliva testing, it is necessary to first review how small a picogram is. A brief summary of weights follows:
1 gram = 1000 milligrams (abbreviated mg)

1 milligram = 1000 micrograms (abbreviated µg)
1 microgram = 1000 nanograms (abbreviated ng)
1 nanogram = 1000 picograms (abbreviated pg).
Therefore 1 gram = 1,000,000,000,000 pg, or expressed inversely; 1 pg = 0.000000000001 g. This, then is approximately the concentration range that an accurate salivary lab test method would need to reach in order to be useful and reliable.

To express this level of measurement in common, every-day terms; consider drinking a cup of coffee in which one lump of sugar (saccharose) was dissolved. After drinking the entire cup of coffee, rinse the empty cup in an olympic swimming pool (50 m in length, 20 m wide, 2 m deep). If the entire contents of the olympic swimming pool were then mixed thoroughly, and one drop was removed from the pool and analyzed with a lab test equal in sensitivity to the saliva test systems, it would theoretically detect and accurately assess the amount of saccharose that was present in that single drop of water from the pool. That waterdrop theoretically contains approximately 1 pg/ml of saccharose .

To further emphasize the overall accuracy of the newer saliva test assays, consider that they are also highly specific and it would be possible to pour into the swimming pool 1 kg of a different sugar (e.g. fructose or maltose) and the assay would still specifically measure the saccharose in one drop without becoming confused by the other closely related sugars which were not saccharose. There are however areas that could possibly introduce false results and it is necessary to exercise precautions to avoid them and obtain reliable test results in the laboratory. These areas will be discussed in following sections.

It will be shown in section 5.2. that there are significant differences in steroid hormone concentrations in blood and in saliva. Despite this, there is an equilibrium between the concentration found in blood and the concentration found in saliva . This equilibrium has been observed to adjust within 1 or 2 minutes of a fluctuation in steroid hormone concentration in blood. In general the level of a steroid hormone in saliva is lower by a factor of between 20 to 100 compared to the level of that same analyte in blood. This factor is influenced by many inputs and varies by different steroid hormone species. Therefore it is not possible to predict the salivary concentration of a steroid hormone by measuring the level in blood and applying an empirically derived correction factor.

3. Saliva Sampling

Saliva samples are very easy to collect compared to blood samples. Sample collection does not require medical assistance, anxiety or significant discomfort to the patient. Even a urine collection requires a place of privacy, which is not essential for a saliva sample. Saliva samples can be collected at home, while travelling, at work, or during physical exercise. As a result it has become possible to investigate new diagnostic strategies which are not easily contemplated with blood samples.

3.1. Sampling Device

Due to the extremely low steroid hormone concentrations in saliva there are special requirements for the selection of an appropriate sampling device. For analytes such as the steroid hormones that are expected to be present in a sample in the pico-gram concentration range, it is possible for several dynamic interactions to influence the analyte. One such interaction is non-specific adsorption of the analyte to certain materials including many plastics that are commonly used for sample collection devices. This adsorption effect is important when collecting saliva, since saliva does not contain the rich protein concentrations found in blood.

Typically, the concentration of proteins found in serum or plasma will coat the surface of any sample collection tube immediately upon contact with the tube. Because the proteins have quickly adhered to the plastic, the steroid hormones in a blood sample are prevented from non-specific adsorption because the proteins have already adhered to it. This high concentration of proteins is not present in saliva samples and so the surface of the plastic sample collection device remains exposed and can interact with the analyte. This is easily demonstrated and documented with certain types of plastic tubes.
Not all of the steroid hormones exhibit the same tendency for adsorption onto plastic surfaces. Progesterone shows a significant tendency for adsorption to many plastic devices and this can result in a significant loss of measurable Progesterone in the saliva sample (more than 50% reduction in

detectable analyte). Polyethylene (PE) plastics have a remarkable affinity for Progesterone, and as a consequence PE plastic should never be used for saliva sampling.

Special precautions are needed to avoid adsorption effects. It is recommended that only a previously validated commercial sampling tube be used to collect saliva samples for steroid hormone lab testing. If that is not possible then it is recommended that the user validate their own collection device. The absorption rate of a sampling device (using Progesterone from a pool of normal male saliva) should not exceed 10%. If the sampling device does not reduce the measurable Progesterone by more than 10%, then that sampling device can be used for saliva sampling.

In the early days of steroid hormone testing, glass tubes were the preferred sampling device, and to some extent this is still true today. In fact glass tubes can be used as a reference for validation of a plastic device. As a note of caution, the stopper can be a weak point in the use of glass collection tubes. Typically the liquid in a sample collection tube will come in contact with the stopper at some point in the process of shipping a sample to a testing laboratory. Because of this, the stopper must also be considered in adsorption validation studies. In many instances, stoppers for glass collection tubes are made of PE and should be rejected for saliva sample containers. Moreover glass tubes are fragile and are not optimal for transporting samples or submitting to a laboratory by use of common carriers or the mail service.

Commercial saliva sampling devices called “Salivettes®” are available on the market, which include a cotton or plastic roll. The roll is inserted in the mouth and held there until it has become saturated with saliva and then removed and placed inside the salivette container. The container is then sent to the testing laboratory. Studies have shown that these rolls can be the source of a variety of interaction effects with regard to the steroid hormones. Some investigators have shown adsorption effects and others have shown false positive lab test results related to the roll in the Salivette. Therefore the use of Salivettes is not recommended for collecting saliva samples for steroid hormone testing.

In summary, validation studies indicate that 2 ml snap-cap tubes made of ultra-pure polypropylene are the container of choice for collecting and submitting saliva samples for steroid hormone lab tests. These tubes come with an integral stopper attached to the tube. The capacity of these tubes is sufficient to accommodate a complete steroid hormone test profile in the laboratory, which can typically be completed with approximately 1 ml of saliva.

3.2. Interference and Saliva

As with blood, saliva samples collected immediately after food intake may cause falsely elevated steroid hormone values. Therefore fasting is highly recommended. Specifically, meat and any milk products may have a strong impact on the measurement of salivary steroids and result in falsely elevated levels.

Many common medications and creams contain high concentrations of steroid hormones, and their use may result in remarkably elevated steroid hormone levels in saliva (or serum). In addition to resulting in elevated levels in serum and saliva, there is also the risk that a patient may inadvertently contaminate a saliva collection device by handling it after applying a cream containing steroid hormones. It is important for a patient using any topical creams to thoroughly wash their hands prior to touching and handling any saliva collection device.

Foaming should be avoided during the saliva collection process. If there is foam present on the saliva in the sampling device, it should be removed. If possible the total volume of saliva in the sampling device should be around 1 – 1.5 ml (disregarding foam). Note however that in most cases 0.5 ml is sufficient for a complete lab test profile.

Special care must be taken concerning blood contamination of saliva samples. As mentioned earlier, the concentration of steroid hormones in blood is between 10 and 100-fold higher than in saliva. Therefore any blood contamination will result in falsely elevated levels in saliva. All saliva samples should be checked for red coloration (evidence of blood contamination), by holding the sample in front of a sheet of white paper and inspecting for any slight indication of reddish tinge in the saliva. If the saliva in the sampling device shows any reddish tinge, then the sample should be discarded and another sample collected after rinsing both the mouth and the contaminated collection device with water. After waiting 10 minutes another saliva sample should be collected. If this sample still shows a reddish tinge then sampling should be done at another time when there is no bleeding in the mouth.

To facilitate this visual inspection process, only colorless ultra-pure polypropylene sample collection devices should be used.

At certain times it is possible that production of saliva in the mouth is insufficient to collect a saliva sample. It is not recommended to chew gum to stimulate the flow of saliva, but an inert object such as a plastic drinking straw may be used. An alternative would be to wait until a time when saliva production is at an acceptable level to collect a sample.

3.3. Stability of Saliva Samples

It is common knowledge in laboratory medicine that serum and plasma samples are rather unstable. As a result, they must be refrigerated or kept cold during both transportation and storage. Saliva samples for steroid testing seem to be more stable. If necessary, saliva samples may be stored at ambient temperature for several days. Saliva samples can be sent to a testing laboratory by routine mail service even in summer without special temperature protection. However, if refrigerator storage is easily available it is desirable even though it is not required. Colder temperatures will always prevent or retard the growth of bacteria which can potentially interfere with accurate lab testing. In contrast to serum or plasma samples, saliva samples may also be frozen and re-thawed repeatedly without causing problems. Repeated freezing and thawing of saliva samples will result in lower viscosity which is desirable in the testing laboratory.

Collection devices and appropriate shipping containers are typically available either from the clinic office or from the testing laboratory. These shipping containers are usually designed so that they minimize any sample stability issues that might arise during the shipping process.

3.4. Saliva Sampling Strategies

Optimal timing strategies for the collection of saliva samples will depend on the diagnostic issue being investigated. Basically there are two strategies: Measurement of the steroid hormone dynamics (fluctuation profile) or measurement of the average hormone level. For either scenario the sampling strategy must take into consideration that steroid hormone secretion will vary noticeably within any given time-frame.
The dynamics of steroid hormone secretion demonstrates variability within:

- daily time-frame
- monthly time-frame
- definable age categories
- gender–specific categories

In addition to the categories listed above, it is possible to see additional episodic variation of steroid hormone secretion within a time-frame of between 1 and 3 hours. This episodic variation is seen in serum and plasma samples as well as in saliva. Therefore, lab testing done on a single sample of either blood or saliva is rarely optimal for establishing a diagnostic tool for the clinician to interpret accurately, because it does not account for the normal episodic variation or any of the time-related factors in the categories listed above. It is important to stress that the investigation of a single blood sample for the concentration of steroid hormones has the same inherent issue of reflecting only an arbitrary result.

Reliable lab test results can only be achieved if multiple sampling strategies are used at the time of collection. The only possible exception to this concept might be Cortisol, the only steroid hormone which could yield clinically useful test results when one single sample is collected and tested. For any of the other steroid hormones, there is no logical alternative to multiple sampling in order to rule out episodic variation. It is unfortunate that the expense, difficulty and level of discomfort associated with venipuncture and blood-drawing procedures have created an expectation at the clinic sites that one single blood sample is a suitable strategy for steroid hormone measurements. As a result, it is commonly thought that a single sample is appropriate for lab analysis, when the reality is that single sampling is a result of the degree of discomfort and difficulty related to multiple blood draws over a limited period of time. With the growing popularity of saliva sampling to test for steroid hormones it is now possible to collect multiple samples which are needed to determine more accurate levels.

3.5 Physiology of Steroid Secretion

What causes the episodic pattern of steroid hormone secretion? The answer to this question can be found in the feedback mechanism which is a function of the hypothalamic/pituitary glands. These glands are a major part of the time-related control of physiological functions in humans (e.g. control of sleep and awakening patterns). The secretion of hormones such as LH, FSH, or ACTH shows an episodic pattern with maximum secretion peaks every 1 to 3 hours. These hormone fluctuations also stimulate the target organs with fluctuating levels of intensity, and the efficiency of target organ stimulation is dependent upon the concentration of the pituitary hormones. Secondary steroid hormone secretion shows time patterns similar to the pituitary hormones. Because steroid hormones are secreted in the free (unbound) form (see section 5.2), the episodic secretion pattern is more exaggerated in saliva than it is in blood. This can be clearly seen in the steroid hormones Testosterone, Progesterone, Estradiol, and DHEA. In Cortisol it is not that obvious, mostly because the concentration of this steroid in saliva is relatively high. Salivary Cortisol assessment will typically reveal a relatively (compared to serum) sharp morning peak and then diminishing values during the day, possibly due to either psychological effects or food intake.

As a result of these potential variables, it is necessary to provide certain minimum patient information to the clinician and testing laboratory in order to enable a proper interpretation of steroid hormone measurements in saliva. The following information is typically required.

- Name and gender of the patient
- Date and exact timing of sampling (each individual sample)
- Time of typical wake-up (not specific to the day the samples are collected). This should be the average wake-up time of the last 10 days. If the wake-up times of the last 10 days has been remarkably irregular, then the sampling should be postponed until a more regular time.
- Premenopausal women: The date of the most recent menstrual period should be noted (first day of bleeding).

The following strategies for saliva sampling are recommended:

a) Time profiles: Used to determine the dynamics of the steroid hormone concentration in saliva. For normal routine assessments a profile may not be needed. For other uses a time profile may yield valuable answers for the professional endocrinologist or clinician. If there is a need for determining a time profile, there are 3 different sampling strategies that may be employed depending upon the requirements of the diagnostic issue. The sampling strategy may cover as little as a 2 hour time period if the episodic secretion pattern is being established (Profile a_1). The sampling strategy may cover an entire day if it is necessary for the clinician to establish a daily profile (profile a_2); The third alternative for sampling strategy covers an entire month if clinically indicated (Profile a_3).

The sampling strategy for profile (a_1) consists of 5 separate collections, spaced over a 2 hour period (1 sample every 30 minutes). Sampling strategy for the day profile (a_2) consists of 5 samples taken throughout the day (used primarily for Cortisol). For this sampling strategy, wake-up time is considered the zero (starting) point, and samples should be taken at 1, 2, 4, 8, and 16 hours after wake-up. A greater number of samples will establish the profile more exactly, but five samples as outlined are considered sufficient. Monthly profile (a_3) strategies are most useful in establishing steroid hormone profiles for premenopausal women. As with the daily profile, more samples over the monthly time-frame will establish the profile more exactly. It is recommended for profile ($a_{3)}$ that multiple samples should be collected during any sampling day, meaning 5 samples within a period of 2 hours (see next section). This should be done after a fasting period of at least 3 hours (sample collection prior to eating is suggested). The first day of the monthly time-frame should be the first day of menstrual bleeding.

b) Measurement of average (mean) values: This is the most cost-effective strategy for steroid hormone testing in saliva, giving sufficiently reliable answers for routine steroid hormone testing. In most cases this strategy will provide useful information to the clinician. Because of the episodic secretion pattern it is recommended that multiple samples be collected over a time-frame of 2 or 3 hours (if indicated) beginning after a fasting period of at least 2 or 3 hours. The last meal prior to the collection period should not contain any food from animal origin, especially milk or meat derived products. For reasons of convenience this collection period could be timed just prior to a meal either in the late AM or in the PM hours before the evening meal (fasting for 2 - 3 hours prior to sampling is recommended). During the 2 hour time-frame for collecting saliva samples, they should be collected every 30 minutes. Modest variation in the collection timing will not be critical, and the collection time-frame can be extended up to 3 hours. It is only important that there is no food intake during this

collection period. Drinking water is allowed and may even be necessary to create sufficient saliva flow. The patient is cautioned to wait 5 minutes after drinking water before collecting a saliva sample. The 5 samples should be collected in separate collection devices. The testing laboratory is responsible for processing these samples and measuring the mean concentration value.

As previously described in section 3.4. the strategy of multiple sampling has greater diagnostic value than assessment of single samples.

4. Quantitative Measurement of Steroid Hormones

Laboratory testing for steroid hormones in saliva requires particular attention to detail because the concentration levels involved are extremely low (picogram range). In this low concentration range any potential variation in laboratory technique can have an exaggerated effect on test results compared to blood testing. As previously explained in section 3.1, the lack of proteins in saliva may result in adsorption to certain plastic surfaces resulting in inaccurate assessment of concentrations. Saliva samples should be collected in carefully selected collection devices, and salivary lab testing should be performed using approved saliva tests which have been developed specifically for this purpose. There are still some laboratories using serum test kits which have been modified for salivary use. The results generated by such modified test kits have been the subject of debate within the scientific community and it is possible that the results of these modified serum assays may not be accurate or reliable.

It is important to note, however, that even the approved saliva test kits available to the testing labs from different manufacturers may generate different results. The reason for this unfortunate phenomenon lies in different calibration processes used by different test kit manufacturers. At this time, there is no international calibration standard available for manufacturers of saliva test kits to use when they prepare test kits for the laboratory market. Because of this lack of a calibration standard, the results generated from different test laboratories are not always comparable if the labs are using test kits from different manufacturers.

It is important that the laboratory report format of any saliva test to the submitting clinician should also provide the reference ranges that have been established in that lab with the use of the test kits that are currently being used to test patient samples. Therefore it is also important for any testing laboratory to use carefully established reference ranges. For most of the steroid hormones these reference ranges should be specific for gender, age, and timing. It is expected that each test laboratory will generate their own reference ranges rather than relying on published data. If steroid concentrations are reported to the clinician together with reliable reference ranges then the interpretation of lab test results will be reliable and useful as a diagnostic tool.

4.1 Sample Preparation

Upon arrival in the testing lab, all saliva samples should be frozen, preferably overnight. The morning the samples are to be tested, they should be thawed and warmed to room temperature. After this, the samples need to be mixed and centrifuged. This should result in a clear supernatant. If the supernatant is not clear the freezing/thawing/centrifugation cycle should be repeated. After that a final visual check for any reddish color will be done to exclude samples with any sign of blood contamination (see section 3.2). If measurement of the mean (averaged) steroid hormone level has been requested, the lab will need to mix equal aliquots from each of the series of saliva samples in a new collection container prior to testing.

4.2 Analytical Systems

There are reliable test kits available for saliva testing which have been developed especially for the determination of extremely low hormone concentrations in saliva. Laboratories should use only test kits that have been deemed suitable for diagnostic use by relevant regulatory authorities. The minimum requirement should be the CE mark (in Europe) or FDA clearance within the US. Laboratories should refrain from modifying test kits which were developed for serum applications. Modified test kits typically are not completely and thoroughly validated and their use has a tendency to generate test results that are less accurate, reproducible or reliable. At this point, there are no automated lab test systems available that were developed for saliva steroid hormone testing. This is not a disadvantage in terms of lab testing accuracy, and it only means that the lab technicians will perform the tests with manual lab equipment, rather than with automated equipment.

4.3. Reporting Lab Test Results

When laboratories report test results to the submitting clinician, it is important to provide a meaningful reference range (normal population range) as well. Depending on which steroid hormone is being tested and reported, appropriate reference ranges may need to be gender- specific, age-range specific, classified as pre-menopausal or post-menopausal, etc. Because the interpretation of steroid hormone measurements in saliva must take into consideration the patient-specific data and also the biological variations within one individual, it is necessary for the patient to provide detailed information at the time the saliva samples are collected, in order for the interpretation of results to be properly compared to the correct reference ranges. This serves to emphasize that steroid hormone measurements from a single sample are not necessarily meaningful in routine laboratory testing. This statement is true for both serum and saliva lab testing, although single measurements are still commonly requested on serum samples because of the difficulties involved in collecting more than one sample in a relatively short period of time.

Interpretation of salivary steroid hormone laboratory test results should take into consideration the following:

a) Cortisol: The secretion pattern of Cortisol will show a characteristic profile with a daily cyclic pattern. During the first 2 or 3 hours after typical wake-up time there is a distinct concentration peak-value. The position of this peak-value is dependent on the average wake-up time of the past week. It is not dependent on the actual wake-up time of the specific day of sample collection (if different from the average wake-up time of the week past). After this concentration peak the Cortisol concentration declines until approximately midnight. Therefore the best time for sample collection to test for the disease named Morbus Cushing (Cushing's Disease)is midnight. Spontaneous increases in Cortisol concentration during the day may occur, commonly due to stress or food intake. Strenuous physical exercise can also result in increased Cortisol concentrations post-exercise. Exercise-induced increase in Cortisol concentration has been reported to exceed the morning peak concentration. After several hours post-exercise the concentration will return to normal levels.
b) Testosterone: The concentration of Testosterone in males is age-dependent. Males between 15 and 30 years of age show the highest values. In a pattern similar to Cortisol, Testosterone secretion shows highest values in the early morning, with peak-values seen before the average wake-up time. Approximately one hour after the average wake-up time, Testosterone concentrations should reach the normal average daytime "level". It has been shown that this daytime "level" is highly dynamic (variable). The secretion pattern of Testosterone in males and females shows significant episodic variation, which may vary from the daytime average values by a factor of 3. If concentration values are averaged by collecting groups of samples rather than single samples at each collection time-point then a decline of Testosterone values by a factor of 2 during the day is typically seen. In females there is no difference between morning and evening values, but episodic secretion patterns (variability) is typical during the course of the day. The measurement of Testosterone levels in the saliva of females has potential application for the diagnosis of hirsutism (6). In this respect the measurement of Testosterone in saliva seems to be superior to other laboratory methods. In adolescents the mean Testosterone concentration continuously rises during maturation. Strong physical exercise also stimulates Testosterone secretion. This exercise-stimulated secretion precedes the Cortisol increase (see previous section). The magnitude of increase is dependent on individual training habits. Post-exercise Testosterone concentrations will return to normal levels or below. Individuals not accustomed to routine exercise will typically show very low values post-exercise.
c) Progesterone: Usually the measurement of Progesterone is done in the second part of the female cycle in order to measure the activity of the corpus luteum. This measurement strategy has value in the determination of infertility. The Progesterone concentration in the second half of the female cycle should be significantly higher than the first part. At the end of the second part of the female cycle the Progesterone drops and causes cyclic bleeding. During pregnancy the Progesterone concentration continuously increases. Similar to Testosterone, Progesterone demonstrates a distinct variable secretion pattern especially in the second half of the female cycle. Therefore multiple sampling is highly recommended to ensure reliable measurement of progesterone. This secretion pattern and multiple sampling strategy is also true for accurate serum measurement. Progesterone concentrations in males and children is in the same magnitude as post-menopausal females.
d) Estradiol: Except during pregnancy Estradiol (17β-Estradiol) is the main estrogene hormone in females. This statement also is true for children and males. Estradiol also shows a distinct

monthly rhythm with maximum values just prior to ovulation. After ferilization Estradiol concentrations rise continuously until term (similar to Progesterone). The basic level of Estradiol in females is significantly higher than levels in children and males. However, after menopause Estradiol levels are rather similar.

e) DHEA: This is the main androgene hormone and is considered to be the precursor of other important androgene hormones such as Testosterone. In blood this hormone is mainly present in conjugated form (DHEA-S) whereas exclusively DHEA is found in saliva. Therefore measurement of DHEA-S in saliva is not medically indicated, except as an indicator of blood contamination. The measurement of DHEA-S in saliva is not related to levels of hormone activity, whereas measurement of the unconjugated form (DHEA) has logical medical application. The concentration of DHEA in saliva is rather similar to that of Testosterone (morning peak, dynamic secretion, day profile etc). Age-dependence and the day profile is even more pronounced in DHEA than in Testosterone.

5. Comparison of hormone testing in blood and in saliva

This section discusses steroid hormone measurement in both saliva and serum. At the time of this writing, the majority of steroid hormone measurement is performed using blood products (serum). This is an interesting observation since there is a growing body of information published in scientific literature suggesting that serum testing may lack diagnostic accuracy, especially at low concentration levels.

5.1. Scientific literature review

In September 2003 a French group of researchers (Taieb et al) published a study regarding validity of the most common commercial methods for the measurement of Testosterone in serum. This study was published in the “Analytical Chemistry” journal(1). Remarkably, the study concluded that none of the 10 commercial serum testing methods was found to be sufficiently reliable for the measurement of Testosterone in children and women. Also remarkably, the editor commented that guessing would be just as accurate as a serum laboratory test. To extend this line of logic; guessing is fast, non-invasive, and inexpensive. Unfortunately, much like serum testing, it isn’t very accurate. Similar results have been published for Estradiol and Progesterone measurements in serum at low concentration levels.

5.2. Physiological dynamics

In order to understand the basic differences in the measurement of steroid hormones in blood and saliva it is important to understand the different physiological forms of these hormones. Most of the steroid hormones in blood are tightly bound to special “carrier” proteins (e.g. CBG, SHBG and others). This bound fraction represents between 95 and 99% of the total concentration of the hormone to be found in the human body. This bound hormone fraction has no hormone activity within the human body. Its only function is to serve as a kind of biological reservoir. We can refer to this as the total hormone concentration. In contrast to this, the hormone activity is exclusively based on the tiny fraction of 1-5% which is not bound to a carrier protein (referred to as free-fraction).

There are no reliable analytical methods available on the commercial market for the measurement of this free fraction in blood products(serum). However this free fraction can be easily measured in saliva, since **only** the free fraction of analyte is found in saliva. The carrier-protein bound steroid hormones are not found in saliva. In order for a laboratory to assess hormone activity (free-fraction) in serum it would be necessary to measure (a)the concentration of the steroid, and also (b)the concentration of the binding (carrier) protein. Following these two lab tests, it would be necessary to apply a mathematical model to arrive at a theoretical estimate of the hormone activity (free-fraction) in serum. This means that we need 2 laboratory measurements plus a mathematical formula in order to yield one theoretical (indirect) estimate of the level of hormone activity.

In addition, serum collection is an invasive process (blood draw) and is not considered to be comfortable for most patients. Consequently, clinicians are reluctant to suggest collecting a closely spaced group of serum samples to compensate for episodic variation (see earlier sections). By comparison, collecting a closely spaced group of saliva samples is not difficult and one laboratory test will yield a result that is relevant and useful to the clinician.

Since clinicians are currently ordering serum tests for steroid hormones, and are only collecting one single sample and are not routinely ordering lab tests for the carrier proteins, it might explain the poor

diagnostic value of steroid hormone measurement in serum. It must be stressed that there is no reliable routine method available for the measurement of biologically active free-fraction steroids in serum. Scientific debate of this subject continues in many forums and it will certainly stimulate thought and action regarding improved sampling and lab testing recommendations (4).

Why is only the free steroid hormone fraction present in saliva? In the human body the blood supply and the salivary glands are separated by a porous membrane. This membrane allows small molecules to pass, provided the molecules are not polar in nature. Therefore very small and non-polar molecules can cross through this membrane by passive diffusion. This phenomenon applies to steroid hormones, which appear first in the blood supply in both carrier-protein bound (inactive) form and biologically active free-fraction. The biologically active free-fraction migrates through the salivary gland membrane, while the inactive carrier-protein bound fraction is unable to do so. As a result, salivary assessment of steroid hormones represents the biological activity and provides a valuable tool for clinical diagnostics.

5.3. Analytical considerations

Steroid hormone measurement in blood (serum or plasma) may also be influenced by interference from various blood components which can render the performance of commercial serum test kits less accurate. The main problem is caused by hormone conjugates which are formed within the body. These are metabolic derivatives of the native steroids linked to soluble molecules (e.g. DHEA-Sulfate in chapter 3.3.e). These conjugates are created by the body to enable the kidneys to excrete the steroids. These conjugates typically can cause specificity problems in commercial serum test kits. In the early days of immunoassays laboratories were forced to separate these conjugates by extraction procedures in order to make the serum tests accurate and specific. Such extraction procedures are not done anymore because the manufacturers of commercial serum test kits and automated blood analysers claim that extraction is no longer needed. Results and conclusions from a variety of scientific publications do not appear to support this conclusion (3). In salivary measurement these conjugates are not a concern since they cannot pass the membrane between the blood supply and the salivary glands. Theoretically, saliva is free from interfering conjugates, and this is a major advantage of salivary testing.

As mentioned in the previous section there are some commercially available serum lab test kits(mainly for Cortisol and Testosterone) claiming to measure free-fraction in serum. Scientific literature does not support this claim (4). There is only one reliable method available for research investigations to measure free-fraction of steroid hormones in serum, and that is symmetric equilibrium dialysis. Symmetric equilibrium dialysis is currently the reference method for measuring free hormone fractions in serum samples.

Unfortunately, this reference method is very complex and expensive and as a result it is not used in routine clinical diagnostics. However, the discussion in the previous section of how only the biologically active free-fraction of steroid hormones in our bodies can pass from the blood supply through the membrane of the salivary gland and into the salivary gland represents a very similar “device” in our body. This “device” in our body mimics much the same function as the laboratory instrument that performs symmetric equilibrium dialysis (4.2.). Measurement of the free-fraction steroid hormones in saliva follows the same principle as the reference method for serum testing.

5.4. Patient dynamics

It is not difficult to collect saliva samples from patients at almost any time or location. This is simply not possible for blood sample collection. Elderly patients, infants, and patients who are not comfortable with blood sample collection procedures are typically not bothered by the non-invasive sampling procedures used to collect saliva samples. In addition, several consecutive samples for measuring the mean value or for detailed profile testing are not a collection problem with saliva samples . Consecutive collection of several samples will yield more valuable results to the clinician compared to a single serum sample.
Blood collection procedures are not only difficult from a logistical standpoint, there is also a level of physical discomfort for infants, anxious patients and people whose veins are difficult to work with which can be easily avoided by use of saliva samples rather than serum samples.
Many laboratory tests other than steroid hormones should logically be performed on serum samples. Clinicians may therefore feel that since they must collect serum samples anyway, they may as well order steroid hormone lab tests on the serum samples as long as they have them. That way they don’t

need to collect additional saliva samples for just the steroid hormone tests. It is unfortunate that this helps to perpetuate the use of serum samples for analysis of steroid hormones, when saliva samples would provide more accurate and viable information to the clinician.

6. Summary

The most reliable method for steroid hormone testing is done with saliva samples. Significant advantages have been detailed in scientific publications It is clear that the patients would benefit not only during the sample collection process but also from improved diagnostic value of the more reliable and accurate laboratory test results. The clinicians would benefit from the superior diagnostic value of saliva measurements as well as a sample collection process that did not depend on the presence of a scheduled appointment with a licensed phlebotomist.

This monograph was written primarily to summarize progress that has been made in steroid hormone laboratory testing. The scientific investigations related to this subject and cited here (see literature citations) have been published over a period of years, and as such their citation and reference in this monograph does not represent blinding new revelations . It is the hope of the author that compiling and commenting on information published by a variety of sources over a period of decades will help to clarify and stimulate all of us to optimize not only our thoughts but our actions as we move forward with all facets of patient management and laboratory testing. If this brochure can contribute to the understanding of medical professionals either in the lab or in clinical practice then the author has accomplished his goal.

It is the author's belief that the time has come to collate existing published scientific information in order to critically review routine laboratory methods for steroid hormone testing as well as patient management/sampling strategies in the clinical setting. Clearly, in an environment where published information implies strongly that guessing would be more accurate than existing laboratory test methods for sera (2), there is a need for laboratorians, scientists and clinicians to understand as much as possible about existing alternatives rather than marching blindly forward doing exactly the same thing we did yesterday. There is also a need for continued research/discussion/input/feedback on this subject and it is the hope of the author that our overall education has only just begun.

Finally, there exists a form of inertia provided by the manufacturers and the users (testing laboratories) of automated analytical systems for measuring steroid hormones in serum. These systems are one of the anchors for "status quo", representing developmental expense and commitment on the part of the manufacturers and hard-money commitment on the part of laboratories who are using them. The very definition of this equation stifles change. These automated systems must be able to speak to questions of whether or not the very samples they test are optimal or whether their recommended samples truly do not require preliminary extraction, as well as questions of their accuracy/reliability at low levels of concentration. Public health, individual patient health, laboratory science and research-oriented biochemistry would all benefit as a result.

Literature appendix:

1. Author: Joelle Taieb, Bruno Mathian, Francoise Millot et.al.
   Title: Testosterone Measured by 10 Immunoassays and by Isotope-Dilution Gas Chromatography-Mass Spectrometry in Sera from 116 Men, Women, and Children
   Published in: Clinical Chemistry 2003 (49:8) Pages 1381 – 1250
2. Author: David A. Herold, Robert L.Fitzgerald
   Title: Immunoassays for Testosterone in Women: Better than a Guess? (Editorial)
   Published in: Clinical Chemistry 2003 (49:8) Pages 1250 – 1251
3. Author: Frank Z. Stanczyk, Michael M.Cho, David B.Endres et.al.
   Title: Limitations of direct estradiol and testosterone immunoassay kits
   Published in: Steroids 2003 (68) Pages 1173 – 1178
4. Author: William Rosner
   Title: An Extraordinarily Inaccurate Assay for Free Testosterone Is Still with Us
   Published in: Journal of Clinical Endocrinology and Metabolism 2001 (86:6) page 2903
5. Author: Charles D. West, Damodar K. Mahajan, Virginia J. Chavré et.al.
   Title: Simultaneous Measurement of Multiple Plasma Steroids by Radioimmunoassay Demonstrating Episodic Secretion.
   Published in: Journal of Clinical Endocrinology and Metabolism 1973 (36 No.6) Pages 1230 – 1236.
6. Author: Josko Osredkar, Ivan Vrhovec, Niko Jesenovec et.al.
   Title: Salivary free testosterone in hirsutism
   Published in: Ann. Clin. Biochem. 1989 (26) Pages 522 – 526
7. Author: Alvin M. Matsumoto, William J. Bremner
   Title: Serum Testosterone Assays – Accuracy Matters (Editorial)
   Published in: Journal of Clinical Endocrinology and Metabolism 2004 (89:2) Pages 520 – 524
8. Author: Christina Wang, Don H. Catlin, Laurence M. Demers et.al
   Title: Measurement of Total Serum Testosterone in Adult Men: Comparison of Current Laboratory Methods Versus Liquid Chromatography – Tandem Mass Spectrometry
   Published in: Journal of Clinical Endocrinology and Metabolism 2004 (89:2) Pages 534 – 543