About ZRT’s Testing Methods

THE SCIENCE OF SALIVA TESTING

Estrogens (estrone, estradiol and estriol), progesterone, testosterone, DHEA-S and cortisol are routinely measured in saliva at ZRT. Why saliva? Steroid hormones in the bloodstream are mostly (95-99%) bound to carrier proteins (hormone-binding globulins, albumin), and in this form they are unavailable to target tissues. Only the unbound fraction freely diffuses into tissues, including the salivary gland. Hormone levels in saliva therefore represent the quantity of the hormone that is currently available to target tissues and actively exerting specific effects on the body. Because of this, salivary hormone levels often relate to specific symptoms of hormone excesses or deficiencies. Research at ZRT has demonstrated clear correlations between salivary hormone levels and reported symptoms. The rationale for and clinical utility of saliva testing is well documented1-13.

The very small concentrations of salivary hormones (only 1 – 5% of the total hormone levels that include protein-bound hormone found in serum) necessitate extremely sensitive assay methods. This is a particular issue for estrogens, which are present in very minute quantities in saliva, especially in older populations such as postmenopausal women. ZRT is unique as the only commercial laboratory using extracted saliva testing for estrogens. Extraction removes contaminants that interfere with the assay and concentrates the sample, significantly improving assay sensitivity compared to the “direct” assay methods available commercially14. In fact, poor correlations between serum tests and non-extraction salivary estradiol assays have unfortunately led to some skepticism about saliva testing. Also, because of the extremely sensitive assays, it is important to avoid blood contamination of saliva as a result of oral injury, therefore toothbrushing must be avoided before collecting saliva for testing15. Saliva testing may also not be appropriate for sublingual hormone users unless samples are obtained at least 36 hours after the last dose. Blood spot testing is a preferred option for these patients.

ZRT Sample Types

Saliva Testing
Saliva testing measures the amount of hormone available to target tissues – the bioavailable amount. For this reason, saliva testing better relates to specific symptoms of excess or deficiency, and is a good option for monitoring hormone therapy.

Dried Blood Spot – p. 4
Blood spot is ideal for measuring hormones and other analytes such as insulin, blood lipids, Vitamin D, thyroid hormones, and elements like lead and magnesium. It offers distinct advantages over serum because it eliminates the need for a blood draw – saving patients time and money.

Dried Urine – p. 6
Urine testing is the best way to measure adrenal and sex steroid hormone by-products and their respective metabolic pathways, providing a gauge for understanding the body’s hormone metabolism. Urine testing is also the preferred method for testing elements like iodine and heavy metals like arsenic and cadmium. Additionally, it’s the most common method for testing neurotransmitters.
Conversely, when some hormones, notably progesterone, are administered topically, saliva levels can rise higher than serum levels\(^{16,17}\). This is because progesterone is carried to target tissues including the salivary glands, where there is rapid uptake and release of the hormone into tissues and saliva, leaving very little hormone in the venous blood returning from the tissues\(^{18}\). Tissue levels of progesterone have been found to be very high after topical progesterone use\(^{19-21}\), and a biological response can be demonstrated, e.g., the reduction of endometrial cell proliferation caused by estrogen therapy\(^{22}\). We have recently published a clinical study showing saliva levels of progesterone increased 10-fold while capillary blood spot levels increased 100-fold compared to levels in venous whole blood and venous serum following application of 80 mg progesterone cream or gel\(^{23}\). This has led us to conclude that when hormones are delivered through the skin or oral or vaginal mucosa, conventional serum hormone tests grossly underestimate hormone delivery to tissues. In contrast, hormone levels in saliva or capillary blood spot better represent tissue hormone uptake.

DHEA-S, the sulfated storage form of DHEA, is measured rather than DHEA because its levels are more stable (DHEA has a much shorter half life in blood) and at ZRT it has been found to correlate very well with reported clinical symptoms. However, as a conjugated hormone that does not diffuse into saliva as rapidly as the unconjugated hormones measured in ZRT’s other hormone assays, its passage into saliva is flow rate dependent\(^{12}\) and therefore flow stimulants such as gum chewing are not advised prior to saliva collection.

Research at ZRT shows good correlations between salivary hormone levels and dosages of hormones given exogenously. Saliva testing is therefore a good option for monitoring hormone therapy and adjusting dosages if necessary.

### Advantages

- Saliva testing, unlike serum tests, measures the bioavailable (“free”) levels of steroid hormones, correlating with symptomatology and potential deficiency
- Samples are collected by the patient at home, allowing convenient timing of collection especially for cortisol, which must be measured at specific times of the day or night
- Convenience of collection allows frequent sampling, e.g., during a menstrual cycle to determine fertility problems
- Hormone levels can be assessed during topical hormone supplementation
- Saliva collection avoids the stress of a blood draw, which can affect levels of cortisol
- Hormones are stable in saliva at room temperature for up to 2 weeks, allowing for worldwide shipment and convenient mailing of samples for testing
- Saliva testing is less expensive than conventional serum testing

### Clinical Utility

Saliva testing can help providers:

- Identify hormone imbalances prior to the appearance of symptoms or disease
- Identify specific hormone imbalances associated with symptoms
- Establish hormone baselines prior to surgery or beginning therapy
- Monitor hormone levels while supplementing, allowing for individualized hormone dosing
- Track patient symptoms and hormone levels using ZRT’s comparative history reports provided with follow-up testing
References


THE SCIENCE OF DRIED BLOOD SPOT TESTING

Dried blood spot (DBS) is an important part of the minimally-invasive hormone testing that is the hallmark of ZRT Laboratory. Blood spot testing was originally developed in the 1960s out of a need to screen newborns for phenylketonuria (PKU), since a simple heelstick is more practical than a conventional blood draw in young infants. Later this was broadened to include tests for congenital hypothyroidism. Today neonatal screening for PKU and thyroid deficiencies using DBS tests is a routine procedure, and assays for a wide range of other analytes in DBS have been successfully developed. The simplicity of sample collection, stability of samples in storage and transport, and excellent correlation of blood spot assays with serum tests, have made it an ideal method for epidemiological and field research studies for a variety of health conditions in both children and adults.

The ability to measure accurately levels of steroid hormones in DBS has important implications for reproductive endocrinology, and also allows effective monitoring of hormone replacement therapy. This is of particular note for sublingual hormone users, for whom saliva testing is not optimal. Hormones held in the mouth as a troche or sublingual drops concentrate locally within the oral mucosa, which results in a higher local concentration in the saliva. This can result in “false high” salivary test results for up to 36 hours, depending on many factors responsible for clearing the locally concentrated hormone from the oral mucosa, including the ability to produce saliva, frequency and types of meals and beverages consumed, and toothbrushing. The blood spot assay circumvents this problem of “false-high” test results seen in saliva of sublingual hormone users because the capillary blood is taken from a site distal to the oral mucosa, the finger.

DBS testing has distinct advantages over conventional serum testing for monitoring topical hormone supplementation. Levels of steroid hormones produced endogenously are remarkably similar in venipuncture serum and finger stick capillary blood spots. However, when hormones are delivered topically (transdermally, sublingually, or vaginally), capillary blood spot levels can be much higher than serum levels. Animal studies investigating tissue uptake of topicaly delivered hormones have shown a striking discrepancy; high tissue hormone levels and much lower serum levels have been observed. Research shows that a physiological dose of 20-40 ng/ml progesterone raises the tissue levels of progesterone to a very high luteal phase level (> 20 ng/g tissue). However, under these same conditions, venipuncture serum progesterone levels only increase marginally to sub-luteal levels (1-3 ng/ml). The same is seen with saliva versus serum levels, with much higher hormone levels seen in saliva. We have recently published a clinical study showing saliva levels of progesterone increased 10-fold while capillary blood spot levels increased 100-fold compared to levels in venous whole blood and venous serum following application of 80 mg progesterone cream or gel. This has led us to conclude that when hormones are delivered through the skin or oral or vaginal mucosa, conventional serum hormone tests grossly underestimate hormone delivery to tissues. In contrast, hormone levels in saliva or capillary blood spot better represent tissue hormone uptake. Using only serum test results to monitor topical progesterone supplementation has led to confusion and can result in over-dosing in an attempt to achieve physiological luteal levels of progesterone.

Sample Collection

Collection of the blood spots is a relatively simple and nearly painless procedure that can be done at home or by the health care practitioner. A simple nick of the finger followed by placing blood drops on a filter card is all that is needed. The kit contains easy step-by-step instructions, skin cleansing wipes, two lancets, a filter paper on which the blood drops are collected, and a band-aid. The dry blood spot sample requires...
no special handling and is returned, together with a requisition form completed by the patient indicating any current hormone therapy and symptoms, to the laboratory for analysis in a prepaid return package. Blood spot samples are collected in the morning before eating or drinking. Topical hormone users should use their hormones daily as usual but avoid applying the hormones with the hands for several days prior to collection.

**Advantages**

- Convenient for both patient and health care practitioner
- No phlebotomist, special preparation such as centrifugation of the blood, or special packaging and shipment required, therefore less expensive and more convenient than conventional blood draws
- Simple and convenient collection of blood at home allows for flexibility of testing at the right time of day or month or following hormone therapy
- Hormones and other analytes stable in dried blood spot at room temperature for weeks, allowing for greater latitude in collection and shipping
- Infectious agents such as HIV are inactivated by drying the blood thus allowing for safer transport and lab testing of the blood sample
- Familiarity of hormone test levels: ranges for hormones in dried blood spots nearly identical to ranges for conventional serum tests

**Clinical Utility**

Dried blood spot testing can help providers:

- Identify hormonal deficiencies or imbalances associated with aging and disease, thyroid dysfunction and symptoms of menopause and andropause
- Link clinical symptoms to specific hormone imbalances identified by the test
- Restore hormonal balance and patient quality of life using test results as a rational basis for treatment
- Monitor patient hormone levels for individualized, physiologic dosing of hormone supplementation
- Track patient progress with comparative history reports provided with follow-up testing

**References**

THE SCIENCE OF DRIED URINE TESTING

Urine testing is a common diagnostic method for a wide variety of analytes. ZRT Laboratory uses dried urine for testing elements (iodine, selenium, bromine, arsenic, and mercury) that directly or indirectly impact thyroid hormone synthesis and intracellular conversion of thyroxine (T4) to triiodothyronine (T3). We have also developed dried urine tests for measuring steroid hormone metabolites, giving doctors a tool to look at hormone bioavailability and metabolic pathways that can highlight risk factors for hormone-dependent cancers. In addition, we offer a test for the primary metabolite of melatonin, MT6s, which is included in both the Sleep Balance Profile and the Complete Urine Metabolites profile. We now offer testing for a range of neurotransmitters in dried urine.

Dried Urine versus Liquid Urine

Collection of urine over a 24-hour period has provided a convenient means to measure the total output of steroid hormones and other analytes during a day. Physiological hormone production fluctuates throughout the day, so hormone testing in a 24-hour urine only provides an average of hormone synthesis, but does not account for peaks and troughs or diurnal variations that occur throughout the day. For example, while total cortisol output can be estimated with a 24-hour urine test, the circadian rhythmic pattern of cortisol production cannot be evaluated with this collection method.

The collection of a 24-hour urine can be messy, and accuracy is dependent on collecting ALL urine in a large plastic jug during the course of the day, accurately measuring the total volume of urine collected, and carefully pouring a small amount of the entire contents of the 24-hour collection into a smaller vial for shipment to the testing laboratory. Urinating directly into a plastic jug is not a simple task for most women, nor is guesstimating the volume collected or pouring a small amount of it from the large plastic jug into a smaller tube. Moreover, collections are often missed, especially those associated with a bowel movement.

ZRT Laboratory has been instrumental in developing a simple, more convenient, and more accurate alternative to liquid urine collection and testing. It involves collecting urine on a filter strip and allowing it to dry. Urine is collected four times throughout the day. The “dried urine” method not only simplifies the collection procedure for patient convenience and compliance but also allows for ease of shipment without concern for sample stability. Urine collection at four time points also overcomes the limitations of interpreting a 24-hour urine collection for hormones that display a well-defined diurnal rhythm (e.g., cortisol and melatonin).

Advantages of Dried Urine Testing Compared to a 24-hour Urine Collection

1. Simple sample collection.
   The collection procedure is simple and can be completed at home. A laboratory-grade filter paper strip is saturated with urine either by dipping it into urine that has been collected in a cup, or by urinating directly onto the strip. It takes very little urine to saturate the strip – only about 2 mL – so it isn’t necessary to have a full bladder before collecting the sample. After saturating the strip, it is then taped to a towel rack or other object from which it can hang down to dry without the strip itself touching anything.

2. Convenience of shipping.
   After at least 4 hours of drying, the sample is ready to be placed in the collection kit box and mailed back to ZRT Laboratory for analysis. No refrigeration is required and shipping costs are low since samples can be sent in the regular mail. This makes shipment to the laboratory more convenient and allows for shipments from anywhere in the world without preservation (frozen
or ice packs). Liquid urine is subject to bacterial contamination, and must therefore be shipped refrigerated or frozen.

3. Sample stability.
ZRT Laboratory runs long-term stability experiments under different environmental conditions for each of the analytes we test. Results of these experiments show that urine dried on filter paper can be left at room temperature for at least a month without compromising test results, and samples can be stored frozen indefinitely. This is an advantage over liquid urine samples, where analytical results may be compromised if there are delays in shipment and/or if the samples are not kept refrigerated or frozen.

4. Multiple collections
Urine can easily be collected at specific time points throughout the day allowing for evaluation of the circadian rhythms of hormones such as cortisol and melatonin. The specific time points required are convenient, avoiding the risk of missed collections.

5. Creatinine correction.
Because urine varies in dilution depending on the time of day or the volume of liquid consumed, all dried urine samples are also tested for creatinine. Creatinine is a natural breakdown product of creatine phosphate in the muscle tissue which is produced at a fairly constant rate. The more dilute the urine, usually as a result of higher liquid intake, the lower the creatinine level. Using creatinine as a correction factor is a very reliable method except in cases of very poor kidney function. All results are therefore expressed in micrograms of the hormone per gram of creatinine, which is more accurate than reporting hormone levels per unit volume, or per 24 hours.

Testing Accuracy
Dried urine has proven to be accurate throughout a wide range of analyte concentrations. Dried urine samples are extracted and analyzed in the laboratory using highly accurate methods including liquid chromatography/tandem mass spectrometry (LC-MS/MS; used for free cortisol, free cortisone, and MT6s), gas chromatography/tandem mass spectrometry (GC-MS/MS; used for steroid hormone metabolites following enzyme digestion and derivatization to increase sensitivity), inductively-coupled plasma mass spectrometry (ICP-MS; used for elements testing) and a direct mercury analyzer for mercury testing. For elements testing, ZRT Laboratory uses external controls with certified analyte concentrations to ensure accuracy.

ZRT Laboratory has carried out extensive validation studies showing that the dried urine test shows an excellent correlation with standard 24-hour collections (see graphs right).
Sample Collection for Elements Testing

ZRT’s elements testing requires only two dried urine samples: the first morning void after waking and the last urine void before bedtime. Our research at ZRT Laboratory has shown that these two samples, when combined, give results quantitatively equivalent to four combined spot collections taken throughout the day (see graphs below).

Unique Advantages of Dried Urine for Cortisol & Melatonin Testing

ZRT has established expected cortisol and melatonin ranges and the four point test results are plotted as seen in the graphs below. The graphs on the left show what is expected for the diurnal levels of urinary cortisol and melatonin (MT6s) in a healthy individual where urinary cortisol is low in the morning, rises several hours later, and then gradually falls to a low point before bed at night. In contrast to urinary free cortisol (UFC), the urinary melatonin metabolite MT6s peaks in the early morning void which reflects the night resting phase. Melatonin then drops throughout the day, reaching a low point in the evening (reflects the greatest exposure to light) and then begins to rise with reduced lighting.

The graphs below are typical of these patterns in a cancer patient where the diurnal cortisol patterns are flat and very little melatonin is produced at night. A distinct advantage of the diurnal urine melatonin versus salivary diurnal melatonin is that in the urine collection the first void is the sum of all melatonin produced during the “dark” phase, when melatonin production should be at its highest. To evaluate salivary melatonin at peak of production requires waking at 2-3 a.m. to collect saliva, which is extremely inconvenient and results in poor compliance.

Sample Collection for Metabolites & Neurotransmitter Testing

The hormone metabolite and neurotransmitter tests require four spot collections: first morning void, second morning void (preferably within 2 hours of rising), evening between 5 and 7 p.m. (prior to the evening meal) and last thing before bedtime. Four collections are required because of the diurnal rhythm of hormone secretion, particularly important for the urinary free cortisol and the MT6s tests, and these time points best portray the expected diurnal patterns of cortisol and melatonin. The four collections are also combined to provide an average of the hormone levels seen in a 24-hour collection. These collections not only capture the peaks and troughs of cortisol and melatonin levels seen throughout the day, but also allow for convenience of collection (first awakening, just before leaving for work, returning from work before dinner, just before bed at night).
Development of Ranges for Steroid Metabolites

ZRT has established specific hormone ranges for gender, menopausal status (premenopausal follicular and luteal; postmenopausal), and exogenous hormone supplementation as it relates to hormone type, dosage, timing, and delivery (e.g., oral, topical, sublingual, patch, etc.). This is particularly important as different methods of exogenous hormone delivery can result in remarkably different levels of the hormone in urine. An example of the variation in the ranges of pregnanediol, as it relates to these parameters, is seen in the diagram below. As seen in this graph, topical progesterone delivery raises urinary pregnanediol very little, while oral progesterone raises pregnanediol levels beyond luteal phase levels.

References