Pros and Cons of Hormone Testing in Different Body Fluids with Different Routes of Hormone Delivery

David T. Zava, PhD
ZRT laboratory
A Guide to Steroid Hormone Testing in Different Body Fluids Following Different Routes of Hormone Administration

<table>
<thead>
<tr>
<th>Type of Body Fluid</th>
<th>None Endogenous Steroids</th>
<th>Oral Steroids</th>
<th>Topical Steroids</th>
<th>Vaginal Steroids</th>
<th>Troche/Sublingual Steroids</th>
<th>Pellet/IM Steroids</th>
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<tbody>
<tr>
<td>Serum</td>
<td>Yes</td>
<td>Yes (1)</td>
<td>No (2)</td>
<td>No (2)</td>
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<td>Yes</td>
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</tbody>
</table>

1) Overestimation: Metabolites interfere with immunoassays
2) Underestimation: Hormone levels not reflective of tissue uptake
3) Overestimation: Requires range adjustment
4) Overestimation: Direct contamination of oral mucosa/saliva
5) Overestimation: Direct contamination of urine
6) Overestimation: IF fingertips contaminated with topical hormones
Endogenous Hormone Production and Testing in Different Body Fluids
ENDOGENOUS HORMONE TESTING IN BLOOD: VENIPUNCTURE SERUM VS CAPILLARY BLOOD (DBS)
Blood Spot and Plasma Levels of Estradiol, Progesterone, LH, and FSH Over a Normal Menstrual Cycle

Validation of Blood Spot Sampling for Gonadotropins and Ovarian Hormone Levels in Reproductive Age Women. Fertility and Sterility, November 2007
A. Edelman, R. Stouffer, D. Zava, J. Jensen

“Lab Tests Made Simple”
Progesterone Blood Spot/Serum Correlation

$y = 1.05x - 0.6$

$R^2 = 0.99$
Blood Spot vs. Serum Correlations

Testosterone

LH

FSH

SHBG
CONCLUSION:
WITH ENDOGENOUSLY PRODUCED HORMONES
VENIPUNCTURE SERUM = CAPILLARY BLOOD (DBS)
ENDOGENOUS HORMONE TESTING IN SALIVA
How do steroid hormones enter saliva?

- As blood circulates around salivary glands, steroid hormones not bound by blood components (i.e., rbc, CBG, SHBG, albumin) freely diffuse through the cells of the salivary gland and into the salivary ducts.
Assay Challenge: Relative Lowest Expected Concentrations of Steroids in Saliva

Relative Concentrations of Saliva Steroids

Minimum Concentration (pg/ml)

- Estradiol: <0.5-1.5 pg/ml
- Testosterone: <0.5-1.5 pg/ml
- Progesterone: <0.5-1.5 pg/ml
- Cortisol: 1000-2000 pg/ml
- DHEA-S: 1000-2000 pg/ml
SALIVARY DIRECT OR EXTRACTION ASSAYS
### EIA/LIA Direct Assay Problems for Estradiol

<table>
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<tr>
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<td>7.6</td>
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<td>47</td>
</tr>
</tbody>
</table>

*Results from Manufacturer of FDA-approved Direct Estradiol Kit*
Direct Assay Precision is Poor Below 2.5pg/ml for Estradiol

Results from Manufacturer of FDA-Approved Salivary Estradiol Kit

“Lab Tests Made Simple”

---

Direct Assay Precision

- **Physiological Range Salivary Estradiol:** <0.5-4 pg/ml

- **True Sensitivity:** Detection Limit = 2.5pg/ml

- **Manufacturer’s Reported Detection Limit:** = 0.3pg/ml
NEAT SALIVA

DHEAS

Cortisol

Progesterone

Testosterone

Estradiol

MATRIX = BACKGROUND
LAB TESTS MADE SIMPLE

EXTRACTED SALIVA

DHEAS

Cortisol

Progesterone

Testosterone

Estradiol

MATRIX = BACKGROUND
ACCURACY, PRECISION, AND RELIABILITY

ACCURATE POOR PRECISION

ACCURATE GOOD PRECISION

INACCURATE GOOD PRECISION
RELIABILITY BASED ON ACCURACY AND PRECISION

- Control Specimens Help Determine The Accuracy And Precision Of A Given Method
- Inter-laboratory Proficiency Studies Help Determine Consistency Among Labs Running Similar Tests
Comparing Estradiol Immunoassays

Wong (Extracted RIA)

Gandia (Extracted RIA)

IBL (Direct LIA)

Chatterton (Direct RIA)
Clinical Utility of Extracted Salivary Estradiol

Do salivary estradiol levels show expected relationships with:

- Menstrual Cycle
- Premenopausal vs Postmenopausal
- Symptoms of Estrogen Imbalance-Deficiency and Excess
- Estrogen Supplementation
**Monthly Cycle**

**Serum Estradiol**

**Salivary Estradiol**

"Lab Tests Made Simple"
Salivary Estradiol: The Menopausal Transition

Estradiol Median vs. Age

n = 49,769

Estradiol Median (pg/ml)

Age

25 30 35 40 45 50 55 60 65 70 75
Salivary Estradiol & Hot Flashes

Estradiol vs. Hot Flash Severity
n = 39,570

Average Hot Flash Severity (0-3)

Salivary Estradiol (pg/ml)

OPTIMAL
REFERENCE RANGE
ENDOGENOUS HORMONE TESTING IN Urine
GC/MS/MS  Testing of Urinary Pregnanediol in Premenopausal Women at Different Phases of the Menstrual Cycle and on Hormonal Contraceptives, and in Postmenopausal Women Supplementing with Topical, Vaginal, and Oral Progesterone.

**Pregnanediol (median)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Level (µg/mg Creatinine)</th>
<th>Median (20-80% Range)</th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td>Follicular Females</td>
<td>152 (92-346) N=24</td>
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<td></td>
</tr>
<tr>
<td>Luteal 36-45 y/o</td>
<td>1324 (849-1932) N=37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal/...</td>
<td>81 (42-171) N=97</td>
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<td></td>
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<tr>
<td>BC Pills</td>
<td>209 (121-2876) N=7</td>
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</tr>
<tr>
<td>Topical Pg Supp</td>
<td>170 (85-403) N=262</td>
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<td></td>
</tr>
<tr>
<td>Vaginal Pg Supp</td>
<td>400 (180-588) N=6</td>
<td></td>
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</tr>
<tr>
<td>Oral Pg Supp</td>
<td>3930 (1965-7373) N=131</td>
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</tbody>
</table>
Urinary Hormone Levels Throughout the Menstrual Cycle

Fig. 1. Mean ± SEM daily urinary gonadotropin and sex steroid excretion patterns in 11 perimenopausal women, aged 43–52 yr (○) compared to those in 11 midreproductive aged women (●). Data are standardized to day 0, the putative day of ovulation, as described in the text. E₁, Estrone conjugates.

<table>
<thead>
<tr>
<th>Reproductive State</th>
<th>ng/ mg Cr PgM/E1M</th>
<th>PgM/E1M ratio</th>
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</thead>
<tbody>
<tr>
<td>Mid-Reproductive</td>
<td>15,000/50</td>
<td>300</td>
</tr>
<tr>
<td>Peri-Menopausal</td>
<td>5000/100</td>
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HORMONE TESTING in Different Body Fluids following Exogenous Hormone Delivery
A Guide to Steroid Hormone Testing in Different Body Fluids Following Different Routes of Hormone Administration

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<td>No (2)</td>
<td>No (2)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Saliva</td>
<td>Yes</td>
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<td>Yes (3)</td>
<td>Yes</td>
<td>No (4)</td>
<td>Yes</td>
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<tr>
<td>Urine</td>
<td>Yes</td>
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<td>No (2)</td>
<td>No (5)</td>
<td>No (2)</td>
<td>Yes (1)</td>
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<tr>
<td>DBS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes (6)</td>
<td>Yes</td>
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HORMONE TESTING IN BLOOD following Exogenous Oral Hormone Delivery
Response to Oral Administration of 100 mg Progesterone (SERUM)

Concentration (ng/ml)

Time after administration (hours)

Immunoassay

LC-MS
Conclusion:

Majority (95+ %) of oral progesterone is rapidly converted to inert progesterone metabolites in the GI tract and liver. These inactive metabolites interfere with serum progesterone immunoassays, resulting in false-high values. Selective extraction methods are necessary to separate progesterone from its metabolites before progesterone can be accurately measured by immunoassay. More accurate testing can be done by extraction-LC/MS/MS methods.
HORMONE TESTING
IN URINE
following
Exogenous Oral
Hormone Delivery
GC/MS/MS Testing of Urinary Pregnanediol in Premenopausal Women at Different Phases of the Menstrual Cycle and on Hormonal Contraceptives, and in Postmenopausal Women Supplementing with Topical, Vaginal, and Oral Progesterone.
Conclusion:

Majority (95+ %) of oral progesterone is rapidly converted to inert progesterone metabolites in the GI tract and liver. These inactive metabolites never enter target tissues as active progesterone and are excreted into urine. LC/MS or GC/MS analysis of orally delivered progesterone indicates very high urinary levels, much higher than luteal phase progesterone. This can lead to the false impression that oral progesterone raises systemic progesterone to levels seen during the luteal phase. Reference ranges should reflect higher levels.
HORMONE TESTING IN SALIVA following Exogenous Oral Hormone Delivery
<table>
<thead>
<tr>
<th>Category</th>
<th>REF RANGE PG (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Premenopausal - Luteal</td>
<td>75-250</td>
</tr>
<tr>
<td>Premenopausal - Follicular</td>
<td>12-100</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>12-100</td>
</tr>
<tr>
<td>Oral Progesterone (100-300 mg)</td>
<td>30-300</td>
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<tr>
<td>Topical, Troche, Vaginal Pg (10-30 mg)</td>
<td>200-3000</td>
</tr>
<tr>
<td>Synthetic Progestins (HRT, Contraceptive)</td>
<td>10-53</td>
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</tbody>
</table>
Conclusion:

Majority (95+ %) of oral progesterone is rapidly converted to inert progesterone metabolites in the GI tract and liver. These inactive metabolites never enter target tissues as active progesterone and are excreted into urine. The salivary gland filters out progesterone metabolites (e.g. progesterone glucuronides and other polar metabolites), thus reducing the level of inert metabolites that can interfere with salivary immunoassays. Thus salivary immunoassays, particularly with extraction, will more accurately reflect the true level of active progesterone following oral progesterone therapy.
Topical Hormone Therapy and Testing in Different Body Fluids
HORMONE TESTING IN SERUM AND SALIVA following Exogenous Topical Hormone Delivery
SALIVA AND SERUM PROGESTERONE LEVELS FOLLOWING 30 MG TOPICAL PROGESTERONE

<table>
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<tr>
<th>TIME OF DAY</th>
<th>SALIVA</th>
<th>SERUM</th>
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<td>17:00</td>
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<tr>
<td>18:00</td>
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<tr>
<td>6:30</td>
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</table>

WEEK 1

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Serum vs. Saliva Steroid Hormone Levels

- Salivary/serum correlations are very good when hormones produced endogenously.
- (salivary hormones = 2-3% of serum hormones)

HOWEVER

- Salivary/serum correlations become problematic with exogenous *topical* hormone replacement.
SALIVA AND SERUM PROGESTERONE LEVELS
FOLLOWING 30 MG TOPICAL PROGESTERONE

HOURS POST APPLICATION

PROGESTERONE (ng/ml)

TIME OF DAY

WEEK 1

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Perceived Saliva Disadvantage?

- TOPICAL application of ALL steroid hormones (Estradiol, Estriol, Progesterone, Testosterone, DHEA, Cortisol, etc.) results in a marked increase in saliva hormone levels but little change in serum hormone levels.
Two Schools of Thought

1. *Serum Camp*: High saliva hormone levels, without concomitant increase in serum levels, represents a concentration artefact in the salivary glands, and hormones are poorly absorbed through the skin.

2. *Saliva Camp*: High saliva hormone levels represents the bioavailable fraction of hormone uptake into tissues, and serum is a poor indicator of hormone uptake through the skin.
HORMONE TESTING IN DRIED BLOOD SPOTS
Blood Spot Progesterone (ng/ml) vs. Serum Progesterone (ng/ml)

**Equation:**
\[ y = 1.05x - 0.6 \]

**R\(^2\) = 0.99**
Venous vs. Capillary Blood Progesterone Levels Following Topical Progesterone Use
Physiological Dosing (20-30 mg) with Topical Progesterone Results in Physiological (Luteal) Levels (15-25 ng/ml) of Progesterone in Capillary Blood

**Blood Spot Progesterone with Topical Supplementation After 8-12 Hours**

- Median Blood Spot Progesterone (ng/ml)
- Daily Progesterone Dosage (mg)

**Optimal Luteal Range**
Reported Serum Range for Topical Pg = 1-3 pg/ml
# Case Example

Patient using 20 mg topical progesterone BID (12 hr last used)

<table>
<thead>
<tr>
<th>Body Fluid Type</th>
<th>Progesterone Level</th>
<th>Luteal Range</th>
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</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.9 ng/ml</td>
<td>3-25 ng/ml</td>
</tr>
<tr>
<td>Blood Spot</td>
<td>24.6 ng/ml</td>
<td>3.3-22.5 ng/ml</td>
</tr>
<tr>
<td>Saliva</td>
<td>1291 pg/ml</td>
<td>75-350 pg/ml</td>
</tr>
</tbody>
</table>

Summary: Capillary blood level of progesterone is 10x serum!
Serum grossly underestimates tissue uptake of progesterone delivered topically
Distribution of Progesterone in Different Body Fluids Following 15 mg Topical Pg

Topical Progesterone Supplementation

- Venous Serum
- Capillary Serum
- Saliva

Hours from 15mg Dosage

Progesterone Concentration (ng/ml)
Endometrial Proliferation with Topical Supplementation

E.P. data from Leonetti F&S 2003

“Lab Tests Made Simple”
What are *Tissue Levels* of Progesterone Following Topical Progesterone Use??

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Placebo (n=8)</th>
<th>Pg (n=7)</th>
<th>E2 (n=9)</th>
<th>E2+Pg (n=9)</th>
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</thead>
<tbody>
<tr>
<td>Pg (ng/g)</td>
<td>0.6+/-0.3</td>
<td>66+/-120</td>
<td>2.1+/-3.8</td>
<td>41.2+/-75</td>
</tr>
<tr>
<td>E2 (ng/g)</td>
<td>0.5+/-0.4</td>
<td>0.5+/-0.7</td>
<td>91.0+/-232</td>
<td>35.5+/-69</td>
</tr>
</tbody>
</table>

"The mean P plasma levels were consistently < 1 pg/ml in each treatment group”

Summary:
- Steroid hormones penetrate through skin and reach tissue sites (only breast?).
- Tissue progesterone levels increase without concomitant increase in plasma Pg.
- Progesterone reduces breast cell uptake of estradiol.
CELL PROLIFERATION MARKERS IN BIOPSIES OF NORMAL BREAST TISSUE FROM WOMEN TREATED TOPICALLY WITH 1.5 mg ESTRADIOL (E2) AND/OR 25 mg PROGESTERONE (Pg).

HORMONE TESTING IN URINE following Exogenous Topical Hormone Delivery
GC/MS/MS Testing of Urinary Pregnanediol in Premenopausal Women at Different Phases of the Menstrual Cycle and on Hormonal Contraceptives, and in Postmenopausal Women Supplementing with Topical, Vaginal, and Oral Progesterone.
URINE TESTING WITH TOPICAL PROGESTERONE

Topical Progesterone with Urine

Average Urine Pregnenediol (ug/mg creat)

Progesterone Dose (mg)
Conclusions:
Progesterone Delivered Topically at a Physiological Dose (10-30 mg):

- Small (1-3x) increase in *venous* serum Pg levels
- Small (1-3x) increase in *urine* Pg (Pregnanediol)
- Large increase (10-100x) in *salivary* Pg levels
- Large increase (10-100x) *capillary blood* Pg levels
- Large increase *tissue* Pg levels
- Invokes *tissue response*—decreases estradiol-induced cell proliferation
Is this true for other steroid hormones or just unique to progesterone?
HORMONE TESTING IN SERUM following Exogenous Topical Estradiol and Testosterone Delivery
Figure 1. Mean serum estradiol concentrations (pg/mL) following topical application of placebo or 1.15 Grams, 2.30 Grams or 3.45 Grams of Estrasorb containing 2.5 mg of estradiol per Gram

Premenopausal Luteal Ovarian Production Ca. 25 ug E2/day
Luteal Serum E2 = 70-150 pg/ml

Micrograms (ug) E2
Applied topically

- 8600 ug E2
- 5800 ug E2
- 2900 ug E2
Ovarian Production = 25-50 ug/day
Evamist Application = 1500-4500 ug/day

Pharmacokinetics parameters for estradiol from one, two, or three 90 mcL sprays of Evamist, as assessed on Day 14 of this study, are described in Table 2.
Serum Testosterone: Monitoring Supplementation

Figure 1
Mean Steady-State Serum Testosterone (±SD) (ng/dL) Concentrations on Day 30 in Patients Applying Testim® Once Daily

“Lab Tests Made Simple”
HORMONE TESTING
IN Saliva and Capillary Blood (DBS) following Exogenous Topical Testosterone Delivery
Salivary Testosterone: Monitoring Supplementation

- Male Salivary Testosterone vs Topical Testosterone After 24 Hours
  - n = 328
- Median Salivary Testosterone (pg/ml)
- Topical Testosterone Dosage (mg)
- Physiological Range

N = 328
Linear Increase in Capillary Blood Testosterone with Increasing Topical Testosterone Dosage

![Graph showing linear increase in blood spot testosterone with increasing topical testosterone dosage. The x-axis represents daily dose of testosterone (mg), and the y-axis represents median blood spot testosterone (ng/dL). The graph shows a physiological range, indicated by a shaded area, and includes a linear trend line.]
Absorption

AndroGel delivers physiologic amounts of testosterone, producing circulating testosterone concentrations that approximate normal levels (298 – 1043 ng/dL) seen in healthy men. AndroGel provides continuous transdermal delivery of testosterone for 24 hours following a single application to intact, clean, dry skin of the shoulders, upper arms and/or abdomen.

AndroGel is a hydroalcoholic formulation that dries quickly when applied to the skin surface. The skin serves as a reservoir for the sustained release of testosterone into the systemic circulation. Approximately 10% of the testosterone dose applied on the skin surface from AndroGel is absorbed into systemic circulation. Therefore, 5 g and 10 g of AndroGel systematically deliver approximately 5 mg and 10 mg of testosterone, respectively. In a study with 10 g of AndroGel, all patients showed an increase in serum testosterone within 30 minutes, and eight of nine patients had a serum testosterone concentration within normal range by 4 hours after the initial application. Absorption of testosterone into the blood continues for the entire 24-hour dosing interval. Serum concentrations approximate the steady-state level by the end of the first 24 hours and are at steady state by the second or third day of dosing.

With single daily applications of AndroGel, follow-up measurements 30, 90 and 180 days after starting treatment have confirmed that serum testosterone concentrations are generally maintained within the eugonadal range. Figure 1 summarizes the 24-hour pharmacokinetic profiles of testosterone for hypogonadal men (<300 ng/dL) maintained on 5 g or 10 g of AndroGel for 30 days. The average (± SD) daily testosterone concentration produced by AndroGel 10 g on Day 30 was 792 (± 294) ng/dL and by AndroGel 5 g 566 (± 262) ng/dL.

50 mg daily = 566 ng/dL
100 mg daily = 792 ng/dL

Endogenous T production by testes = 3-5 mg/day
Topical Hormone Delivery

Are we underestimating and overdosing?
CONCLUSIONS

In this population of older men with limitations in mobility and a high prevalence of chronic disease, the application of a testosterone gel was associated with an increased risk of cardiovascular adverse events. The small size of the trial and the unique population prevent broader inferences from being made about the safety of testosterone therapy. (ClinicalTrials.gov number, NCT00240981.)
FDA Drug Safety Communication: Ongoing safety review of Evamist (estradiol transdermal spray) and unintended exposure of children and pets to topical estrogen

Safety Announcement

[07-29-2010] The U.S. Food and Drug Administration (FDA) is reviewing reports of adverse effects from Evamist in children who may have been unintentionally exposed to the drug through skin contact with women using this product. FDA has also received reports of inadvertent exposure in pets.

Evamist contains estradiol, an estrogen hormone. It is used in women to reduce hot flashes during menopause. Evamist is a topical product, sprayed on the skin on the inside of the forearm between the elbow and the wrist.

Patients should make sure that children are not exposed to Evamist and that children do not come into contact with any skin area where the drug was applied. Women who cannot avoid contact with children should wear a garment with long sleeves to cover the application site.

Children unintentionally exposed to Evamist may experience premature puberty. Female children may experience nipple swelling and breast development. Male children may experience breast enlargement.

Pets exposed to Evamist may exhibit signs such as mammary/nipple enlargement and vulvar swelling. FDA is currently reviewing these reported adverse events and is working with the company to identify
Why is this so important to understand?
Need to have a hormone test that accurately reflects how much hormone is being taken up by tissue, not how much is circulating in venous blood.

With topically delivered hormones, venipuncture serum and urine grossly underestimate (at least 10x) tissue uptake of hormone. Underestimating tissue uptake, based on venous serum results, often leads to overdosing.
Prevents overdosing of patients with topically delivered hormones and allows for a more physiological delivery of hormones to target tissues.

Opens new doors to the use of topical progesterone for treating progesterone-deficiency states (e.g. infertility and hyperplasia of breast and endometrium).
For more information on Topical Progesterone and Testosterone see:

www.zrtlab.com/professionals/zrt-blogs/entry/testosterone-elixir-or-dangerous-drug and
Percutaneous progesterone delivery via cream or gel application in postmenopausal women: a randomized cross-over study of progesterone levels in serum, whole blood, saliva, and capillary blood

Joanna Y. Du, MD,1 Puy Sanchez, MD,1 Lila Kim, BS,1 Colleen G. Azen, MS,2 David T. Zava, PhD,3 and Frank Z. Stanczyk, PhD1,4

Abstract

Objective: This study aims to investigate the distribution of progesterone in venous whole blood, venous serum, fingertip capillary blood, and saliva after its topical application in both cream and gel formulations.
### A Guide to Steroid Hormone Testing in Different Body Fluids Following Different Routes of Hormone Administration

<table>
<thead>
<tr>
<th>Type of Body Fluid</th>
<th>None Endogenous Steroids</th>
<th>Oral Steroids</th>
<th>Topical Steroids</th>
<th>Vaginal Steroids</th>
<th>Troche/Sublingual Steroids</th>
<th>Pellet/IM Steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Yes</td>
<td>Yes (1)</td>
<td>No (2)</td>
<td>No (2)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Saliva</td>
<td>Yes</td>
<td>Yes (3)</td>
<td>Yes (3)</td>
<td>Yes</td>
<td>No (4)</td>
<td>Yes</td>
</tr>
<tr>
<td>Urine</td>
<td>Yes</td>
<td>Yes (1)</td>
<td>No (2)</td>
<td>No (5)</td>
<td>No (2)</td>
<td>Yes (1)</td>
</tr>
<tr>
<td>DBS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes (6)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

1) Overestimation: Metabolites interfere with immunoassays  
2) Underestimation: Hormone levels not reflective of tissue uptake  
3) Overestimation: Requires range adjustment  
4) Overestimation: Direct contamination of oral mucosa/saliva  
5) Overestimation: Direct contamination of urine  
6) Overestimation: IF fingertips contaminated with topical hormones
Lab Tests Made Simple

Interstitial space
Capillary bed
Venule

Lymphatic capillary
Tissue cell
Arteriole

Endothelium of lymphatic capillary
Interstitial fluid
Opening
Lymph
Anchoring filament