Salivary Estriol as an Index to Fetal Wellbeing
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We adapted two commercially available kits for measuring serum estriol to use in assaying salivary estriol in the third trimester of pregnancy. Salivary estriol increases during gestation, as does unconjugated estriol in serum. With a meticulous collection protocol, fetal wellbeing can be monitored as successfully with saliva samples as with serum. A fetal demise was charted similarly by the salivary and the serum estriol assay. Inter- and intra-assay reproducibility (CV) of the saliva assay was 6.2%. Day-to-day CV, based on a fasting sample before breakfast but obtained 1 to 3 h after rising, was 14.8% (SD 5.8%). Values for salivary estriol obtained immediately after ingestion of food or drink dropped by 19.0% (SD 17.9%) and 32.1% (SD 21.9%), respectively, as compared with values determined immediately before intake of food or water.

Additional Keyphrases: fetal status: saliva vs serum as sample: variation, source of: radioimmunoassay

During the past 15 years the ability to measure estriol in biological fluids during the third trimester of pregnancy has progressed from the assay of milligrams of estriol per gram of creatinine in urine, to micrograms of estriol per liter of serum, to nanograms of estriol per liter of saliva. The possibility of substituting salivary estriol assays for assay of unconjugated estriol in serum to assess fetoplacental wellbeing has piqued some interest (1–6). Salivary sampling can facilitate the serial, daily collection of estriol data by eliminating repetitive venipunctures. This is of particular value for nonhospitalized patients, patients whose veins have been compromised, and some patients living in rural areas.

Most, if not all, of the salivary estriol assay procedures currently found in the literature are based on tritiated-label or extraction methods, or both. We report a simple, direct technique involving an iodinated label, and evaluate its performance with a random sample of healthy normal persons as well as high-risk cases in the third trimester of pregnancy. Its use in one case of fetal demise is documented.

Materials and Methods
Reagents and Protocols

We used two different kits for the study. Most of the work was performed with an estriol [¹²⁵I] kit ("KIANEN," NEA-062; New England Nuclear (NEN), North Billerica, MA 01862), a kit requiring extraction when used for assay of unconjugated estriol in serum. To demonstrate that the method does not reflect the idiosyncrasies of a specific kit, we also assayed standards for two standard curves and two saliva samples with the more recently developed NEN direct [¹²⁵I]-estriol kit (cat. no. NEA-097). We used the Micropartition system with YMT membranes (Amicon Corp., Danvers, MA 01923) and [2,4,6,7-³H(N)]estriol to evaluate the amount of protein-bound estriol in saliva. Biofluor (NEN) was used as a scintillation cocktail. To determine the amount of conjugated estriol, we hydrolyzed saliva specimens with the enzyme solution (containing glucuronidase and sulfatase) from the "Cost-a-Count" estriol kit (Diagnostic Products Corp., Los Angeles, CA 90045).

With the minor modifications noted below, we followed NEN's directions for the serum estriol assay with the KIANEN kit (NEA-062) to assay estriol in saliva. Because the estriol species in saliva is not protein bound, we omitted the extraction step. Thus the salivary estriol assay begins with the radioimmunoassay protocol.

We set up 11 pairs of polypropylene or glass tubes as follows:

- Tubes 1 and 2, for 100% bound, receive in the order listed: 400 µL of buffer, 100 µL of tracer, 500 µL of solid-phase second antibody, and 100 µL of antiserum (first antibody).
- Tubes 3 and 4, for nonspecific binding (NSB), are prepared similarly but with 500 µL of buffer and no antiserum.
- Tubes 5–16 are used for preparing a standard curve covering the following estriol concentrations: 50, 100, 250, 500, 1000, and 2500 ng/L. These tubes receive 200 µL of buffer and 200 µL of each standard solution, plus tracer and first and second antibodies.
- Tubes 17 and 18 are "total counts" tubes; tubes 19–22 are designated for two controls. For the latter we use two pre-analyzed saliva samples, one of which is chosen to read near 500 and the other near 1000 ng/L. Controls are asporrised into polypropylene tubes, frozen, and stored at –20 °C. Buffer for these tubes is increased to 300 µL, to which 100 µL of control saliva is added. Everything else is exactly as for the "100% bound" tubes.
- Samples are set up in duplicate as described for the controls, i.e., 100 µL of saliva, 300 µL of buffer, 100 µL of tracer, 500 µL of solid-phase second antibody, and 100 µL of anti-estriol antiserum.
- The contents of all tubes are vortex-mixed and then incubated on ice (4 °C) for 1 h. This is followed by a 10-min centrifugation at 2000 × g at 4 °C. The supernate is aspirated and the radioactivity of the pellet counted in the gamma counter.
- Serum/plasma estriol is distributed between conjugated and unconjugated forms (7). The distribution of salivary estriol between the conjugated and unconjugated forms was determined by comparison of enzymatically hydrolyzed samples vs the same samples assayed unhydrolyzed. Identification of salivary estriol as free or protein-bound was evaluated by incubating overnight at 4 °C, in capped polypropylene tubes, 250 000 counts/min of [³H]estriol with 300 µL of saliva or 300 µL of isotonic saline for protein-bound and nonspecifically bound estriol, respectively. For separation we ultrafiltered the samples through an Amicon YMT filter system. We added 50 µL of filtrate to 10 mL of scintillation cocktail (Biofluor) and counted the radioactivity in a beta counter. The percentage of free estriol was calculated from the recovered saliva counts/min divided by the filtered total counts.

The NEA-097 kit, unlike its predecessors, is designed to measure unconjugated estriol in serum "directly," i.e., without a preparatory extraction. To prepare the two standard curves with the NEA-097, we followed the manufacturer's

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 Received June 21, 1985; accepted October 16, 1985.
instruction exactly, except that we added either 200 μL of phosphate-buffered saline (10 mmol/L, pH 7.4) containing 1 g of gelatin per liter (PSGB) or 200 μL of normal saliva from a nonpregnant woman to each tube, including the tubes for NSB, total counts, and standards. Samples are assayed in this system by adding 100 μL of 0.00 standard, 200 μL of saliva sample, the manufacturer’s recommended 500 μL of tracer/separato r, and 200 μL of antiserum.

Sample Collection and Storage

The saliva samples were collected without stimulation of flow, i.e., without giving subjects such items as chewing gum or citric acid before collection. The subjects collected about 3 mL of saliva in a sterile polypropylene screw-cap tube, which was then stored at −20 °C until assay. Before assay, the samples were thawed, centrifuged, and decanted into 12 × 75 mm polypropylene tubes.

Populations

Salivary estriol was studied in two consenting populations. The first group (A) was 41 high-risk patients who were being monitored by determinations of serum estriol in the obstetric clinic. Samples from these subjects were obtained randomly, no special collection protocol having been established regarding prior food or beverage intake. Saliva samples were obtained immediately after blood was drawn for serum estriol. Reviewing these data prompted us to set up a second study (population B) involving four different schemes for saliva collection, in an attempt to develop a protocol that would minimize short-term fluctuations in salivary concentrations of estriol.

Population B consisted of four groups of consenting, normal, outpatient clinic subjects in the last trimester of pregnancy (Table 1). The first group of 10 fasting subjects (B-1) collected two samples of saliva 20 min apart, ate a breakfast of their choice in the hospital cafeteria, and on return to the Ob/Gyn unit collected saliva. Two more saliva samples were collected 20 and 40 min later. Blood samples were drawn at the clinic on arrival, i.e., with the first fasting saliva collection, after breakfast, and 30 min thereafter (halfway between the 20-min and 40-min postprandial saliva sampling).

The second group (B-2), 11 subjects, collected saliva as follows: one sample while fasting, then one within 10 min after breakfast and every 20 min thereafter for 120 min.

The third group (B-3) of 10 subjects collected saliva samples on five consecutive mornings, upon rising, before taking any food or drink. On the fifth morning the subjects came to the clinic and collected a second preprandial sample, drank 250 mL of water, and collected saliva within 5 min of drinking the water, then every 20 min for the next 80 min.

The last group (B-4) of 11 subjects collected saliva samples on five consecutive mornings between 07:30-09:00 hours in the following protocol: they drank 250 mL of water immediately upon rising, abstained from food, liquids, gum, candy, or cigarettes for the next hour, and then collected the saliva sample. They stored these samples at home in the freezer until bringing them to the clinic on their next visit.

Results and Discussion

The compartmental distribution of estriol between serum and saliva is as follows. There is virtually no protein-bound estriol in saliva, as determined by overnight incubation at 4 °C of [3H]estriol with saliva followed by ultrafiltration: 99.8% (SD 1.6%) of the [3H]estriol was recovered in the filtrate. Whereas about 92.5% of serum estriol is conjugated, only 20% (SD 16.8%) (n = 18) of the salivary estriol is conjugated, as determined by enzymatic hydrolysis of total salivary estriol. The amount of unconjugated, free estriol in serum is 16.0% (SD 2.5%; n = 30) of the total unconjugated estriol found in serum, that in saliva is 11.4% (SD 2.5%; n = 30) of the total unconjugated estriol in serum.

Assay Characterization

The assay as described for NEA-062 yields a useful standard curve between 10 and 500 pg of estriol per assay tube. Normal salivary estriol concentrations in the third trimester, based on our experience as well as that of others (1–6), range from 400 to 5000 ng/L.

Assay characteristics of the antiserum were accepted as reported by New England Nuclear Corp.; important cross-reactivities include 70% for estriol sulfate, 0.5% for 17β-estradiol, and 0.1% for estriol-16a-glucuronide.

Interassay reproducibility, evaluated in 10 and eight consecutive assays of controls containing 853 and 2020 ng of estriol per liter, respectively, was 6.2% and 8.6% (CV). Intra-assay CV, based on duplicate assays of 75 patients’ samples, was 6.2%.

We diluted saliva samples with equal volumes of assay buffer to evaluate parallelism between the standard curve and sample dilutions (Table 2). Differences between 100- and 50-μL aliquots of saliva assayed in the same run averaged 1.27%; i.e., the curve for samples paralleled that obtained with standards. The improved reproducibility (over the 6.2% quoted for intra-assay reproducibility) is probably ascribable to the relatively high estriol concentrations of the samples assayed by dilution.

To verify that salivary estriol may also be assayed with commercial kits that differ substantially from NEA-062, we produced two standard curves by using NEA-097 (Figure 1). The two curves are superimposable; i.e., saliva does not alter the matrix sufficiently to affect the assay. Protein-containing buffer (0.00 standard or PSGB) is essential in any system for measuring salivary estriol; otherwise, estriol may be lost through adsorption to the assay tube. For two saliva samples assayed in duplicate, once each with the NEA-062 and the NEA-097 kits, the results were 3678, 1446, and 3558, 1496 ng/L, respectively.

Saliva Collection

The details of the saliva collection turned out to be more important than anticipated. The correlation between serum

Table 1. Population B Characterization

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age, yr</th>
<th>Gestational stage, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-1</td>
<td>10</td>
<td>18-26</td>
<td>33-41</td>
</tr>
<tr>
<td>B-2</td>
<td>11</td>
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<tr>
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<tr>
<td>B-4</td>
<td>11</td>
<td>19-40</td>
<td>35-40</td>
</tr>
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</table>

Table 2. Effect of Sample Volume on Estriol Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Saliva vol assayed, μL</th>
<th>Estriol, ng/L</th>
<th>% variation between samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>2586</td>
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</tr>
<tr>
<td>2</td>
<td>50</td>
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</tr>
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<td>3</td>
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<tr>
<td>5</td>
<td>100</td>
<td>4918</td>
<td>0.92</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>4826</td>
<td>1.29</td>
</tr>
</tbody>
</table>
The mean estriol concentrations in saliva samples obtained from subjects in group B-3 immediately upon rising in the morning varied from those in a later preprandial sample collected as soon as the subjects were seen at the outpatient clinic, by an unacceptable 34.1% (SD 35.3%; n = 10). The day-to-day variation for samples obtained immediately on rising in the morning (B-3) varied by 23.6% (SD 14.7%). Day-to-day variation based on a fasting sample obtained 1–3 h after rising (B-4) varied only by 14.8% (SD 5.8%; n = 9). The standardized collection described for group B-4 under Populations, but modified to a collection of 1 mL of saliva, was eventually adopted as the preferred routine protocol.

Variations of salivary estriol concentration collected at 20-min intervals for 80 min after food or water intake resemble those encountered in an earlier study of unconjugated serum or plasma estriol (Figure 3) (9). Similarly, salivary estriol increases greatly during gestation, as does unconjugated estriol in plasma (Figure 4) (9).

**A Case of Fetal Demise**

In the case of one of the 41 high-risk patients, a 23-year-old primagravida, obese class A diabetic patient, the fetus died. The patient had been diagnosed as a gestational diabetic on 1/10/83 on the basis of an abnormal 3-h glucose tolerance test result. As of 1/20/83, she was considered to be under good control on a 1900-kcal (7965-kJ) diet, her 2-h postprandial concentration of glucose in serum ranging from 850 to 1300 mg/L.

On 3/21/83, approximately five days past term, she had a nonreactive nonstress test. Fetal movement charted by the mother was less than 10 movements per hour. Her cervix was long and closed.
Arrangements were made for an oxytocin challenge test at 08:00 hours the following day. When the patient arrived at the Labor and Delivery Unit, fetal heart sounds were not heard and she reported that fetal movement had diminished the previous evening. After ultrasonography, a diagnosis of intra-uterine fetal death was made (3/22/83). A 4139-g stillborn male infant was delivered on 3/26/83.

In retrospect the fetal demise was heralded equally accurately by either the salivary or the plasma assay (Figure 5). Unfortunately, results by neither assay had decreased sufficiently from 3/14/83 to 3/17/83 to 3/21/83 to warrant anything but moderately increased alertness by the physician and the laboratory. Our standard criterion for alerting the physician-in-charge to potential fetal distress has been a 40% decrease in successive determinations of concentrations of serum estriol (7) or, alternatively, a decrease of 40–45% from the mean of three preceding values (10).

Comments

Salivary unconjugated estriol reflects serum (or plasma) unconjugated estriol concentration (11), although the concentrations in saliva are much lower than those in serum. Approximately 84% of the unconjugated estriol in blood is protein bound; thus, the salivary:serum estriol equilibrium depends on the 16% of free (as opposed to protein-bound) unconjugated estriol found in serum. Current RIA procedures are sensitive enough to assay this low estriol concentration accurately, provided that a buffer containing enough protein to prevent estriol losses to adsorption is included in the protocol.

Half of the salivary assays recently reported in the literature involve use of a tritium label (1, 2, 4), which precludes widespread use of the procedure because few clinical laboratories have access to a scintillation counter. Two of the assays that involve $^{125}$I-labeled estriol (5, 6) are based on use of in-house-produced antisera, and the Amer sham unconjugated $^{125}$I-labeled estriol kit (3) requires extraction of 200 $\mu$L of saliva with 2 mL of ether before the assay. The advantage of our protocol is that the assays do not involve extraction and are based on commercially available antisera and $^{125}$I tracers.

The salivary concentration of free unconjugated estriol (11%), compared with total unconjugated serum estriol concentration, corresponds well with that found by Vining et al. (6) and with the 9.83% predicted by Dunn et al. (12) from a computer simulation.

The conditions under which the saliva samples are collected have not been addressed in previous papers, with the possible exception of a study by Nguyen et al. (13). As was also reported by Vining et al. (6), morning samples obtained immediately on rising are less reproducible than those obtained later in the day. Indeed, the variability is such as to make useless saliva specimens collected immediately on rising. As shown in Figure 2, the effects of food and water intake make a standard collection protocol necessary. If such a procedure is followed, then a decrease in salivary estriol concentration greater than 40–45% from the mean of the three preceding values should be viewed with alarm, just as is the case for fluctuations in the concentration of estriol in serum (7). That is, a second sample should be obtained promptly and the patient observed closely until the decision to deliver the baby can be made or the salivary estriol concentrations return to the previous values. Although the patient whose case we report had not received specific instructions regarding saliva collections, her blood was being drawn (and saliva collected) 2 h postpartum for determinations of serum glucose. The fact that her salivary estriol concentrations reflected her fetoplacental status accurately is probably, in part, a function of the accidental collection protocol imposed by her diabetic state. Actually,
this case is the second fetal demise documented by monitoring of salivary oestriol (14).

As we have previously shown (8), there is no circadian rhythm to the concentration of unconjugated oestriol in serum or plasma, so none is to be expected in its concentration in saliva (8). Vining et al. (6) also were not able to show any consistent, significant fluctuation of salivary oestriol concentration between 06:00 and 22:00 hours in a study in which samples were collected hourly.

We conclude that it is possible to monitor fetal well-being by determining the concentrations of oestriol in saliva. This noninvasive technique for obtaining the daily specimens is ideal for monitoring high-risk pregnancies. Where hospitalization and (or) daily venipuncture is not otherwise justified, the method is feasible and will give the desired information.

\textbf{BIANLEN} Oestriol $^{125}$I RIA kits were a gift from New England Nuclear Corp.

\textbf{References}


