Automated Continuous-Flow Radioimmunoassay for Salivary Estriol

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We have automated a radioimmunoassay for salivary estriol by using the "Southmead" continuous-flow system. The assay, based on a well-validated manual assay, involves a Sepharose-coupled antiserum and an ¹²⁵I-radioligand. The antibody-bound and free radioligand fractions are separated by on-line filtration. Performance of the automated assay has been reliable during six months of regular use, and compares favorably with that of the manual assay. Drift (0.5 nmol/L per 100 samples) and sample carryover (3%) are acceptable, and the throughput rate is 55 samples per hour. The system is suitable for the assay of the large numbers of samples generated by daily saliva specimen collection in the third trimester of pregnancy.

Additional Keyphrases: pregnancy • screening • fetal status

The measurement of plasma unconjugated estriol during late pregnancy is widely used in the assessment of fetal well-being. While the importance of daily, rather than weekly, monitoring has been emphasized (1, 2), the routine collection of daily blood specimens is not practicable. The measurement of salivary estriol offers the advantages of a simple collection procedure that can be carried out by the patient herself, and it is therefore feasible to routinely construct profiles of day-to-day estriol concentrations throughout the third trimester of pregnancy (3-6).

We recently described a rapid manual radioimmunoassay (7), which we used for a preliminary study of salivary estriol in normal and abnormal pregnancies (8). This assay has been adequate for determination of daily samples from selected groups of high-risk patients, but the implementation of a program of wider screening demands an automated assay system. We report the development of an automated radioimmunoassay for salivary estriol, implemented with the continuous-flow automated assay system (the "Southmead System") described by Ismail et al. (9).

Materials and Methods

Equipment

A detailed description of the principles and construction of the "Southmead" automated system has been given elsewhere (9). Conventional continuous-flow technology is used to achieve precise and accurate aspiration and mixing of sample, radioligand, and antiserum. The air-segmented reactant stream is then incubated for a pre-determined period by passage through a series of mixing coils. The antiserum used is coupled to Sepharose-gel beads, and separation of the antibody-bound and free radioligand fractions is by on-line filtration.

The equipment is compact, flexible, and relatively inexpensive, because most of the modules are commercially available. We used a T40 Sampler (Hook and Tucker Instruments Ltd., Croyden, U.K.), a Type HP 30 peristaltic pump (Horstmann Gear Co. Ltd., Bath, U.K.), and a Grants JB1 water bath (Grants Instruments, Cambridge, U.K.). Mixing coils were from Advanced Medical Supplies, Aldershot, U.K., and the nylon mesh filter (Monodur PES 10) was from Verseidag GmbH, Kenpen, F.R.G. Peristaltic valves were Joucomatic 22700092 240v two-way valves (Air Control Products, Brailington, Bristol, U.K.). Gamma radioactivity was counted in a coil of polythene tubing set in the well of a scintillation counter (Model 112 F12/MD13, with a 40 x 40 mm well; Nuclear Enterprises, Sighthill, Edinburgh, U.K.). The electronic control unit and counter were constructed as described previously (9).

Flow-rated pump tubing and transmission tubing were from Technicon Instruments Co. Ltd., Basingstoke, U.K. Figure 1 shows the manifold for the automated radioimmunoassay of salivary estriol.

Each 700-μL sample is aspirated for 60 s by a conventional proportioning pump, followed by a 5-s wash, and mixed on-stream with solid-phase-bound antibody and radiiodinated ligand. After incubation for 12 min the reaction mixture is directed to the separator block, where the bound radioligand is separated by forced filtration across a highly permeable nylon-mesh membrane. Flow through the system is controlled by an arrangement of four valves that enables the solid-phase to be collected beneath the membrane, washed with saline, and then rapidly eluted (for gamma counting) to the coil placed in the well of a scintillation counter. The operation of the counting and valve-switching sequences is triggered by the detection of dye (bromoresol green) in the inter-sample wash stream.

Reagents

The automated radioimmunoassay for salivary estriol is based on a manual method that, together with the procedures for raising sufficient quantities of appropriate antiserum and preparing the radiodinated ligand, has been described previously (7).

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Estriol, histamine, Brij 35 detergent, stock brom cresol green solution (7 g/L of 0.1 mol/L NaOH solution), and all steroids used for determination of cross reactivity were from Sigma (London) Chemical Co., London, U.K.

Na\textsuperscript{125}I (cat. no. IMS-30) was from Amersham International plc, Amersham, U.K.

Bovine serum albumin (BSA), Cohn Fraction V, was from Armour Pharmaceuticals Ltd., Eastbourne, U.K. Except as noted, all other reagents ("Analar" grade) were from BDH Chemicals Ltd., Poole, Dorset, U.K.

**Assay buffer.** This was phosphate-buffered saline (10 mmol/L, pH 7.4) containing 1 g of BSA and 1 g of sodium azide per liter.

**Solid-phase coupled-antibody suspension.** The solid-phase rabbit antiserum, prepared as previously described (7) and diluted 500-fold with assay buffer, was stable on storage at 4 °C for at least six months. It was diluted to 4000-fold before use, with assay buffer containing 1 mL of Brij 35 detergent solution per liter. During each assay run the Sepharose-bound antiserum was kept in suspension with a magnetic stirrer.

**Radioligand.** Estriol-3-(carboxymethyl) ether was prepared by the method of Rao and Moore (10) and coupled to histamine, previously iodinated with \textsuperscript{125}I by a Chloramine-T procedure, substantially by the method of Hillier and Read (11). The radioligand, stored in ethanol at 4 °C, was stable for at least one 60-day half-life of \textsuperscript{125}I. Stock estriol-3-[\textsuperscript{125}I]iodohistamine radioligand was diluted in assay buffer to give 60,000 cpm (nominal\textsuperscript{1}) per 100 μL.

**Estriol standards.** A stock solution of estriol, 100 mg/L of ethanol, was stored at 4 °C; standards were prepared weekly. A 100-μL aliquot of the appropriate dilution of the stock solution in ethanol was evaporated under nitrogen, the residue was dissolved in 10 mL of assay buffer, and the solution (buffer stock) was allowed to equilibrate for 1 h. Appropriate aliquots of the buffer stock were diluted to 20 mL with assay buffer to give working standards with 0, 25, 50, 100, 150, 200, 300, 400, and 500 pg of estriol per 100 μL of assay buffer.

**Diluent for forced filtration.** This diluent was saline (20 g of NaCl per liter) containing 5 mL of Brij 35 detergent solution per liter.

**Wash/dye solution.** Stock brom cresol green solution was diluted 10-fold with assay buffer containing 1 mL of Brij 35 detergent solution per liter.

**Priming mixture.** Pooled male saliva was diluted with an equal volume of phosphate-buffered saline (10 mmol/L, pH 7.4) containing BSA (25 g/L), sodium azide (1 g/L), and Brij 35 detergent solution (1 mL/L).

**Procedures**

**Preparation of saliva specimens.** Collect unstimulated whole saliva (at least 3 mL) into 5-mL disposable polystyrene blood-collection tubes and store frozen at -20 °C. Thaw specimens at room temperature and, after centrifugation at 1000 × g for 20 min, decant the clear supernates for assay or storage at -20 °C. This procedure produces a fluid less viscous than whole saliva and thus with more favorable flow characteristics.

**Assay procedure.** To start the analysis, pump de-ionized water through the reagent- and sample-lines for 10 min, followed by priming mixture for 10 min. Start pumping solid-phase antibody and radioligand, and aspirate assay buffer from 10 sample cups to allow the system to settle. Aspirate the standards in duplicate, then duplicate samples of assay buffer, and then duplicate low-, medium-, and high-concentration quality-control pools. Aspirate sample specimens, with duplicate quality controls (preceded by duplicate buffer samples) every 20 cups to assess drift throughout the assay run.

Any abnormal results are apparent upon visual examination of the recorded output. Rerun samples with abnormal results at the end of the assay run. If significant drift (>1.0 nmol/100 samples) is detected through the run, then take appropriate remedial action and repeat any doubtful analyses.

At the end of each run, wash all reagent lines with sodium hypochlorite solution, 0.5% (available chlorine), followed by de-ionized water.

**Data reduction.** Determine the estriol concentrations in saliva specimens by interpolation from the dose–response curve by a log-logit transformation.

**Results**

**Dose–response curves.** Using the automated protocol, we generated a series of six dose–response curves. The composite standard curve so obtained was compared with that obtained for six dose–response curves obtained with the manual radioimmunoassay and the same reagents (Figure 2).

The curves were superimposable, 50% of the radioligand binding at zero dose being inhibited by an estriol concentration of 5 nmol/L. By the automated system, the sensitivity of the dose–response curve, defined according to Kaiser and Specker (12) as the least amount distinguishable from zero at the 95% confidence level, was 0.07 nmol/L. Log-logit

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\footnote{The mass of radioligand was kept constant throughout the life of the preparation. Thus 60,000 cpm "nominal" is that dilution corresponding to 60,000 cpm on the day of preparation.}

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**Fig. 2.** Replicate dose–response curves obtained for the automated assay (○) and the manual assay (□)

Lower curves (and right-hand ordinate) show precision profiles for computed analyte concentrations obtained by the automated assay (●) and by the manual assay (△)
regression analysis of the standard curve data gave a correlation coefficient exceeding 0.998.

Calculation of the precision of computed analyte concentrations for six replicate determinations of standards showed similar profiles for the manual and automated assays, with CVs of <12% over the normal range for estriol in the third trimester of pregnancy.

Carryover was determined, according to the criteria of Broughton et al. (12), by computing the effect of three consecutive high samples upon the estriol concentrations measured in three consecutive low samples. The root mean square of the carryover (3.0% range −1.4% to 5.0%) was measured in 11 sets of alternating groups, and was similar to that reported by Ismail et al. (9) for the automated radioimmunoassay for plasma thyroxin with the “Southmead” system (range −1.8% to 3.4%).

Drift. We assayed in a single run 90 samples of a pool of saliva specimens collected during the third trimester of pregnancy. The mean concentration of estriol measured in the pool was 3.99 nmol/L (CV = 6.0%). Table 1 summarizes our analysis of the means and CVs of the measurements obtained for nine successive groups of 10 samples. Least-squares regression analysis of these means vs the number of samples assayed gave a correlation coefficient of 0.75 and a regression line of slope −0.5 nmol/L per 100 samples. The variation due to drift through the run (CV = 5.7%; n = 90) was similar to that due to intra-assay variation. Drift was assessed for seven successive routine runs by least-squares regression analyses performed on quality-control samples placed at intervals through each run. Mean estimates of drift for low, medium, and high pools were 0.2, 0.4, and 0.9 nmol/L per 100 samples respectively.

Assay throughput. The sampling rate is 55 cups per hour, which, after a standard curve has been run, gives a specimen throughput rate of 42 cups (21 duplicate specimens) per hour. During a 6-h run, 220 specimens (110 duplicate specimens) may be assayed (duplicate quality-control specimens are included after every 20 cups).

Assay precision. Saliva collected from normal women in the third trimester of pregnancy was used to prepare saliva pools containing low, medium, and high concentrations of estriol. Replicate samples (n = 20) from each pool were assayed in a single run, with single samples placed in random order (determined from tables of random numbers) throughout the run. The results obtained for the assay of these pools—the within-assay variation together with the effect of any carryover between the samples—showed CVs of 7.5% to 9.6%. No significant drift was observed throughout the run.

A precision profile was constructed for estriol concentrations measured in duplicate saliva specimens (n = 41) assayed in a single run. The effects of intersample carryover were eliminated by arranging the samples in the run so that short sequences of singlet samples (each sequence preceded by a low-pool quality control sample) were each immediately followed by a repetition of the same sequence of samples. Effects due to drift, if present, were minimized by restricting repeated sequences to 10–15 samples. The precision profile for the automated assay (Figure 3) was calculated by the method of Ekins (14), and indicates that, over the working range of the assay (1–15 nmol/L), the mean CV for duplicates is less than 9%.

An analysis of variance (15) was applied to the dose estimates obtained from the assay of samples of three quality-control pools in 14 successive runs. Duplicate samples of low-, medium-, and high-concentration quality-control samples (preceded by duplicate buffer samples) were placed after the standard curve and after a run of at least 20 saliva samples. The between- and within-batch components of the total variance for all batches are shown in Table 2.

Specificity of the antisera was assessed according to the criteria of Abraham (16). The antisera used in the system shows a high cross reactivity with estriol-3-sulfate and estriol-3-glucuronide. Cross reactivity with estriol-16-glucuronide and other steroids of physiological importance is low.

Comparison with the manual method. Saliva specimens (n = 42) from women in the third trimester of pregnancy were measured by the manual method (7) and by the automated method. The results (range 1–12 nmol/L) correlated well,
Deming's analysis (17) giving $r = 0.980$, with a regression line of slope 1.03 and an intercept of 0.09 nmol/L.

**Matrix effects.** Although half the reactant cocktail is saliva, we observed no significant matrix effect. Saliva specimens (range 6.2–15.5 nmol/L) collected during late pregnancy from five women were serially diluted with assay buffer, and the estriol concentrations in the resulting dilutions were measured with the automated assay. Data were "normalized" by expressing measured estriol concentrations as a percentage of the concentration in the undiluted pool (calculated from the least-squares regression line). The regression lines had a mean slope of 1.02 (range 0.99 to 1.04) and a mean intercept on the ordinate (percentage of the estriol concentration in the undiluted pool) of $-1.4\%$ (range $-0.1\%$ to $-4.5\%$).

**Preparation of saliva specimens.** Freezing samples overnight is not suitable for treating specimens for which a same-day return of results is required. Although freezing and thawing specimens more rapidly does not produce the dense, gelatinous precipitate characteristic of overnight storage, the resulting supernates have low viscosity. We therefore investigated the effect of more rapid treatment procedures on salivary estriol concentrations.

Samples from a pool of saliva specimens, freshly collected from women in the third trimester, were subjected to a variety of freezing and thawing procedures. Samples subjected to same-day freezing and thawing were assayed in a single run. Samples stored overnight (either at 4 or $-20^\circ C$) were assayed in another single run. Different methods of treating freshly-collected saliva specimens did not significantly affect measured estriol concentrations (Table 3).

**Discussion**

The routine collection of daily saliva specimens throughout late pregnancy must be coupled with an assay capable of giving a rapid return of results on large numbers of specimens. A cost-effective solution to this problem has been achieved by the use of the "Southmead System," which has proved reliable during six months of regular operation and has given results that compare well with those of a well-validated manual assay (7).

The nylon-mesh filter provides a robust, cost-efficient means for separating the bound and free radioligand fractions. The valve-switching sequence provides a "back flushing" phase during each collection/wash/elution cycle, so that clogging of the mesh is virtually eliminated. A single separator block may be used for several months without receiving any attention.

The automated assay consumes rather large volumes of saliva, approximately 700 mL per sample. At least 3 mL of saliva specimen must therefore be collected to provide sufficient supernate for assay in duplicate. However, the assay of single rather than duplicate samples was considered to be acceptable when specimens were being assayed as part of a series through pregnancy. A repeat assay of the remaining supernatant saliva would be done when there is evidence of departure from the trend of an individual profile or if the result is outside the normal range.

The use of saliva rather than plasma for determination of unconjugated estriol has the advantage that a preliminary extraction step is unnecessary, the salivary estriol being mainly in the unconjugated form (7, 18). Furthermore, it is relatively easy to raise sufficient quantities of suitable antiserum, because high specificity with respect to estriol conjugates is not necessary. Although the current automated assay is based on an estriol-6-BSA/estriol-3,125I-radioligand system, we have found that the use of antiserum to estriol-3-BSA (which cross react with estriol-3 conjugates) will give sensitive assay systems with high reliability.

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**References**


**Table 3. Effect of Post-Collection Treatment on Estriol Concentrations Measured in Saliva**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean estriol, nmol/L</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>Freezing</td>
<td>Thawing*</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>2.9</td>
</tr>
<tr>
<td>Rapid</td>
<td>Rapid</td>
<td>2.9</td>
</tr>
<tr>
<td>Rapid</td>
<td>Slow</td>
<td>3.0</td>
</tr>
<tr>
<td>3 h—(20°C)</td>
<td>Rapid</td>
<td>2.9</td>
</tr>
<tr>
<td>3 h—(20°C)</td>
<td>Slow</td>
<td>2.8</td>
</tr>
<tr>
<td>Overnight (20°C)</td>
<td>Rapid</td>
<td>2.7</td>
</tr>
<tr>
<td>Overnight (4°C)</td>
<td>None</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Rapid = 37°C; slow = 4°C.
* All results are the mean of six determinations.
Enzyme Immunoassay of Free Thyroxin in Serum

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We describe a double-antibody enzyme immunoassay for free thyroxin (FT4) in serum with use of β-d-galactosidase conjugated to thyroxin. The method is uninfluenced by thyroxin-binding globulin or albumin. Values for FT4 so determined correlated well with those determined by radioimmunoassay (r = 0.98) and equilibrium dialysis (r = 0.89). The mean variability (CV) within and between assays was 7.4% and 7.6%, respectively. The measurable range of FT4 in serum was 2.8 to 109 ng/L. The FT4 concentrations in serum as determined by this method were 8.4 to 15.5 ng/L for 26 normal adult subjects; 26 to >109 ng/L for 10 patients with hyperthyroidism; <2.8 to 8.0 ng/L for seven patients with hypothyroidism; 7.3 to 15.8 ng/L for eight pregnant women; and 12.2 and 13.5 ng/L for two patients with low concentrations of thyroxin-binding globulin.

Additional Keyphrases: nonisotopic immunoassay • thyroxin-binding globulin • pregnancy • hyperthyroidism • hypothyroidism • β-galactosidase • thyroid status • reference interval

Measurement of free thyroxin (FT4), considered the physiologically active form of thyroxin (T4) (1), is essential for evaluating thyroid function, particularly in conditions in which thyroxin-binding globulin (TBG) is altered.2 FT4 assay is also useful for mass screening for neonatal hypothyroidism (2) and postpartum thyroid dysfunction (3).

Various methods have been used to estimate FT4; each has its advantages and disadvantages. Equilibrium dialysis (4) is technically complicated and has limited clinical applicability. Measurement of the FT4 index (5), widely used routinely, is an indirect method. Recently, a simple radioimmunoassay (RIA) for detecting FT4 was developed, and several commercial RIA kits for FT4 are now available (6). However, the labeled T4 analog used in these kits is bound to serum albumin, so the FT4 values measured by these methods are influenced by the serum albumin concentration and must be cautiously interpreted when the patient has an abnormal albumin concentration (7). Weetall et al. (8) reported an enzyme immunoassay for FT4 in which T4 was conjugated to horse-radish peroxidase. This conjugate did not bind to TBG but did bind to serum albumin.

Here, we describe a new enzyme immunoassay for FT4 in which we used a conjugate of T4 and β-d-galactosidase that is not influenced by either TBG or albumin.

Materials and Methods

Materials

Reagents: 4-(Maleimido-methyl)cyclohexane-1-carboxylic acid succinimide ester was obtained from Zieben Chemical Co., Ltd., Tokyo, Japan; l-T4 (free acid) and o-nitrophenyl-β-d-galactopyranoside were from Sigma Chemical Co., St. Louis, MO 63178; β-d-galactosidase (EC 3.2.1.23, from Escherichia coli, 5 g/L) was from Boehringer-Mannheim, Mannheim 31, F.R.G.; Bio-gel A-5m was from Bio-Rad Laboratories, Richmond, CA 94804; rabbit IgG and human albumin were from Miles Laboratories, Inc., Elkhart, IN 46515; rabbit antiserum to T4, goat antiserum to rabbit γ-globulin, purified TBG, rabbit antiserum to TBG, and a T4 RIA kit were from Eiken Immunocological Co. Ltd., Tokyo 114, Japan; and an Amerlex® FT4 RIA kit was from Amersham International Ltd., Amersham, Bucks., U.K.

T4-galactosidase conjugate: The conjugate was prepared by the method of Yamamoto et al. (9) and Yoshitaka et al. (10), with modifications. We mixed 50 μg of T4 in 50 μL of dimethylformamide with 10 μg of 4-(maleimidomethyl)cyclohexane-1-carboxylic acid succinimide ester in 5 μL of dimethylformamide, then incubated this for 90 min at 30 °C, stirring every 5 min. We then added 10 μL of a 1 mol/L glycine solution, to yield the T4-maleimide.

We centrifuged the β-d-galactosidase solution (0.2 mg/40 μL) at 2000 × g for 5 min and dissolved the resulting pellet in 0.8 mL of phosphate buffer (0.1 mol/L, pH 7.0) containing 1 mmol of MgCl2 per liter. After adding 150 μL of the T4-maleimide solution to this, we incubated the mixture for 30 min at 30 °C, and then applied all of it to a 1.5 × 45 cm column of Bio-gel A-5m that had been equilibrated with the phosphate buffer containing 1 mmol each of MgCl2 and mercaptoethanol per liter. We eluted the column with the same buffer and collected 1-mL fractions.

To 100 μL of each fraction (diluted 150-fold) we added 200 μL of anti-T4 antiserum (diluted 6000-fold with 10 mL/L...