
A Rapid Method for Repurifying Partly Degraded Radiolabeled Steroids

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This rapid method for repurifying radiolabeled steroids from degraded products is based upon differential partitioning of intact and degraded molecules between aqueous-phase solution and anhydrous diethyl ether. Results compare favorably with those by liquid-chromatographic methods.

The rate of degradation of radiolabeled steroids—and therefore their useful life as analytical reagents—is determined in part by the conditions of storage such as temperature and solvent phase (1, 2). Before use, radiolabeled steroids in organic solvent are usually repurified by extensive chromatography, to eliminate contaminating steroids and degradation products. Radiolabeled steroids degrade more readily in aqueous solutions, which diminishes their usefulness for accurate and precise analytical studies. When such degradation becomes excessive, the radiolabeled steroids in aqueous solutions are usually discarded. We report here a rapid method of repurifying representative tritiated and iodinated steroids from degraded products. The method is based upon the differential partitioning of intact and degraded molecules on extraction of their aqueous solutions with anhydrous diethyl ether.

Materials and Methods

Apparatus

We used a "high-pressure" liquid-chromatography system that included a Model U6K injector, a Model 440 absorbance detector, a 20.7 MPa (3000 lb/in.2) pump, and a C-18 µBondapak reversed-phase column (all from Waters Associates, Inc., Milford, MA 01757), and a "Alpha 200" fraction collector (Buchler Instruments, Fort Lee, NJ 07024). All other equipment was described previously (3).

Reagents

All reagents were analytical grade. Tritium-labeled cortisol (lot no. NET-396), estradiol (lot no. NET-013), testosterone (lot no. NET-370), and progesterone (lot no. NET-332) were obtained from New England Nuclear, Boston, MA 02118; radiiodinated estrone (lot no. D-1140) was a gift from Micromedics, Horsham, PA 19044; and radiiodinated estradiol (lot no. 1180C) was a gift from Nuclear Medical Systems, Inc., Newport Beach, CA 92663. The sources of radio-inert steroids and antiserum, the reagents, and the preparation of phosphate-buffered isotonic saline containing gelatin and dextran-coated charcoal suspension have all been reported previously (4, 5). The anhydrous diethyl ether used for extraction was from Mallincrodt, Inc., St. Louis, MO 63147. Absolute methyl alcohol was from Fisher Scientific Co., Fair Lawn, NJ 07410. The water was deionized, filtered through charcoal, and doubly glass-distilled. Liquid scintillation cocktail ("Insta Gel") was from Packard Instrument Co., Downers Grove, IL 60515.

Tritium-labeled steroids were initially purified on Celite (Fisher Chemical Co., Cincinnati, OH 45242). Chromatography and stock solutions containing 100 000 to 200 000 cpm/100 µL were prepared in phosphate-buffered saline containing gelatin. Working solutions with a radioactivity of 10 000 cpm/100 µL were prepared by diluting the stock solution with the assay buffer.

Procedures

In our laboratory, stock solutions of radiolabeled steroids are repurified when the nonspecific binding relative to total count equals or exceeds 5%. The procedure is as follows.

Transfer the stock solution of radiolabeled steroid to a 22 × 175 mm glass tube. Add five volumes of anhydrous diethyl ether and vortex-mix for 2 min. Allow the tubes to stand for 5 min at room temperature; then freeze the aqueous phase at −40 °C and decant the organic phase into another 22 × 175 mm glass tube. Evaporate the ether under nitrogen at 50 °C and redissolve the residue in 5.0 mL of phosphate-buffered saline. Measure the radioactivity and adjust to appropriate working concentrations. Again determine the nonspecific binding relative to total count.

For the purpose of these experiments, the residual (i.e., extracted) aqueous phase was also placed in a 50 °C water bath and the remaining organic solvent was evaporated.
under nitrogen. The nonspecific binding of this residual solution is also reported.

In these experiments, nonspecific binding was determined as follows. In duplicate 10 × 75 mm glass tubes, incubate 100 μL of radiolabeled steroid solution (adjusted to a radioactivity of 10,000 cpm per tube) with 600 μL of phosphate-buffered saline for 1 h at 37 °C, followed by 1 h at 4 °C. Then add 200 mL of cold, continuously stirred dextran-coated charcoal suspension to each of the nonspecific-binding tubes. Vortex-mix the contents of all tubes for 5 s, and place them in an ice bath for 15 min. Then centrifuge all tubes at 4 °C for 15 min at 3000 × g. Carefully decant the supernates into liquid-scintillation vials containing 8 mL of cocktail, thoroughly mix, allow to equilibrate for 30 min, and count the radioactivity in each vial for 5 min. Then compare the counts for the nonspecific-binding tubes with those for the total-count tubes.

The protocols for radioimmunoassays were similar to those previously described (5).

To verify this method, we used liquid chromatography, and progesterone as the representative analyte. Partly degraded, repurified, and residual tritiated samples, each adjusted to approximately 10,000 cpm per 100 μL, were chromatographed as follows. Add 600 μL of each designated sample and 400 μL of distilled water to separate test tubes containing 1 μg of radio-inert progesterone. Vortex-mix all tubes to dissolve the radio-inert ligand and to give an injection solution containing approximately 6000 cpm and 100 ng of progesterone per 100 μL. The mobile phase consists of methanol/water (3:2 by vol), at a pressure of 8.6 MPA (1250 lb/in.²) and a flow-rate of 1.0 mL/min. Using a microsyringe, inject 100 μL of each designated sample and collect the effluent in an automatic drop fraction collector in 1-mL (70-drop) aliquots. Transfer each fraction to a liquid scintillation vial containing 10 mL of cocktail. Obtain the total count by adding 100 μL of designated solution to 900 μL of mobile phase and transferring this solution to vials containing scintillation cocktail. Mix the contents of each scintillation vial, allow the mixture to equilibrate, and count the radioactivity for 5 min.

**Results and Discussion**

As shown in Table 1, the nonspecific binding, representing degradation of the tritiated and iodinated steroid molecule, is significantly decreased (p < 0.001, paired t-test) after extraction with organic solvents. Evidently the intact steroid molecule partitions into the organic phase and the degraded products remain in the aqueous phase as reflected by the high nonspecific binding in the residual aqueous phase, a presumption that is further confirmed by the results of the liquid chromatography of progesterone (Figure 1). The solution containing partly degraded compound (Figure 1A) has a very polar component (peak no. 1) of degraded progesterone relative to the nonpolar component (peak no. 4), which coincides with the peak for the radio-inert progesterone. After extraction with anhydrous diethyl ether, the polar component is essentially no longer present in the repurified radiolabeled steroid solution (Figure 1B) but is in the residual solution (Figure 1C). The relative size of the peaks at position 1 agree with results for nonspecific binding for progesterone in Table 1. Peaks 2 and 3, relatively consistent in all three preparations, could indicate either intermediary products of progesterone degradation or some unidentified impurities in the preparation of parent compound. We chose a stable, nonpolar steroid molecule such as progesterone as a model in these experiments because the degraded radiolabeled products were most readily identified. Similar data were obtained for other steroids (Table 1) under modified chromatographic conditions.

Gelatin must be present in the phosphate-buffered saline in monitoring the degradation of radiolabeled steroids. In its absence, no apparent change in nonspecific binding or degradation of radiolabeled steroid can be detected when these methods are applied; nonspecific binding remained <2% of the total count when gelatin was deleted from the phosphate-buffered saline. Apparently, degraded radiolabeled steroid, but not the intact steroid, is bound to the gelatin and is not adsorbed by the dextran-coated charcoal.

The duration of extraction is also important. If the extraction period exceeds 5 min even degraded radiolabeled products are partitioned into the organic phase.

This method does not distinguish isomers of the same steroid.

**Table 1. Nonspecific Binding of Radiolabeled Steroids as Percentage of Total Count**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Before extraction</th>
<th>Extracted</th>
<th>Residue</th>
</tr>
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<tbody>
<tr>
<td>[3H]Cortisol</td>
<td>6.5</td>
<td>1.0</td>
<td>39.5</td>
</tr>
<tr>
<td>[3H]Testosterone</td>
<td>6.1</td>
<td>1.6</td>
<td>34.0</td>
</tr>
<tr>
<td>[3H]Estradiol</td>
<td>5.0</td>
<td>1.1</td>
<td>28.6</td>
</tr>
<tr>
<td>[3H]Progesterone</td>
<td>6.7</td>
<td>1.0</td>
<td>42.9</td>
</tr>
<tr>
<td>125I-labeled estradiol</td>
<td>10.6</td>
<td>2.3</td>
<td>58.0</td>
</tr>
<tr>
<td>125I-labeled estrone</td>
<td>12.5</td>
<td>3.1</td>
<td>66.1</td>
</tr>
</tbody>
</table>

*All total counts were adjusted to 10,000 cpm/100 μL per tube.*
compound nor can it be used as a substitute for chromatography to isolate specific steroids.

In summary, this is rapid (20 min) and practical method for the economical repurification of radiolabeled steroids. It can be applied to both tritiated and iodinated steroids. Results compare favorably with those after various chromatographic methods that require more manipulations, more personnel time, and expensive equipment.

References

CLIN. CHEM. 30/1, 120–121 (1984)

Salivary Estriol Concentrations during Normal Pregnancies, and a Comparison with Plasma Estriol

J. J. Evans, A. R. Wilkinson, and D. R. Alickin

Saliva would have advantages over plasma or urine for monitoring estriol during pregnancy. Specimen collection, after stimulation of flow by citric acid, is non-invasive and simple. We measured concentrations of unconjugated estriol in saliva and compared them with those in plasma in normal pregnancies, and found a good correlation (r = 0.79). In addition, trends of concentrations in saliva and plasma were statistically compared and found to be highly correlated. The variation among individuals in the saliva/plasma concentration ratio suggested that some inter-individual factor(s) may affect this relationship. The normal reference interval for unconjugated estriol concentration in saliva from 20 weeks of gestation to term was established.

Additional Keyphrases: reference interval · concentration ratio, saliva/plasma · fetal status

The measurement of plasma estriol during pregnancy is widely used for monitoring fetal well being. However, urine collections for this purpose are inconvenient for the woman and are not always reliable and accurate, and venepunctures for blood assays require clinic visits and can be stressful. On the other hand, collection of saliva is non-invasive and can even be done at home. We have previously shown in a small number of women that the concentration of unconjugated estriol increases in saliva as pregnancy progresses (1). Here, we extend those findings and compare estriol concentrations in saliva and plasma.

Subjects and Methods

We studied 50 women who attended the antenatal clinic at Christchurch Women's Hospital before 25 weeks of gestation (as estimated from the last menstrual period and usually confirmed with at least one measurement of fetal biparietal diameter). The women had clinically normal pregnancies. We collected into plain glass tubes about 2 mL of saliva, stimulated by placing on the tongue a small square of dry filter paper that had been soaked in citric acid. This technique has been previously demonstrated not to affect the concentration of estriol measured, as compared with that in unstimulated specimens (1). After centrifuging the saliva to remove sedimentable material, the supernate was decanted and stored at −20 °C. Ordinarily, we collected saliva and plasma together between 0700h and 1000h; occasionally this was not possible, but the correlation between concentrations of estriol in plasma and saliva appeared not to be altered by the increased time intervals.

Estriol in saliva was measured by RIA as previously described (1), which briefly is as follows. An ether extract of saliva was evaporated, the residue was redissolved in borate buffer, and two aliquots were assayed by mixing with a solution of antiserum [raised against estriol-6-(O-carboxymethyl)oxime–bovine serum albumin; Steraloids Inc., Wilton, NH 03086] and tritiated estriol in borate buffer containing bovine serum albumin. Antibody-bound and free fractions were separated by precipitation with saturated ammonium sulfate. Plasma samples were assayed similarly (2), except that the volume of ether phase evaporated after extraction was less. Standard curves of appropriate ranges were constructed.

Results

We established the range of salivary estriol concentrations for each week of gestation throughout pregnancy (for weeks 20 through 40) and calculated the mean concentrations (Figure 1).

Comparing the corresponding concentrations of estriol in saliva and plasma, we found good correlation (r = 0.79) between 320 pairs of saliva and plasma specimens from 50 women. For 18 women with r >0.90 who had five or more paired samples collected during their pregnancies, the plasma to saliva concentration ratio ranged from 6.1 to 14.0. Therefore, the ratio of estriol concentrations was often relatively constant for a given woman, but was not constant from woman to woman. The range of correlation coefficients for 39 women with five or more paired samples was 0.99–0.38 (mean 0.81); there were only three women for whom we observed poor (r <0.50) correlations.