New Extraction Method for Radioimmunoassay of Serum Estradiol

Peter Bonne Eriksen

A new extraction procedure is applied here to radioimmunoassay of serum estradiol. Small columns packed with porous, grainy kieselguhr are loaded with the samples. The kieselguhr material acts as support for the water phase, from which the lipophilic compound estradiol is eluted with diethyl ether. With use of a specially constructed rack, several columns can be handled simultaneously, which makes the procedure convenient for routine use: 40 columns can be processed in about 30 min, including application of samples, elution, and evaporation of solvent. The results correlate well \( (r = 0.98) \) with those by conventional liquid–liquid extraction, and the new method has lower interassay coefficients of variation. When tritiated estradiol in water solution was applied onto the columns, 97.7% was recovered in the extract. The current cost of materials used for the extraction is about $0.20 per column.

Additional Keyphrases: hormones • column extraction vs liquid–liquid extraction

The value of determining 17\(\beta\)-estradiol in serum, alone or in combination with determination of other hormones, has been recognized for several years. Data on estradiol are important in assessing disorders of the menstrual cycle or for monitoring induction of ovulation \( (1, 2) \). In early pregnancy, a low serum estradiol concentration is an indicator of threatened abortion \( (3, 4) \). Like other steroid hormones, estradiol is usually measured by radioimmunoassay after extraction or chromatographic purification from serum \( (5) \). A few years ago a new solid-phase extraction technique was developed for the assay of drugs in biological samples \( (6) \). Columns of porous, finely divided kieselguhr particles adsorb the serum or urine samples, acting as carrier for the water phase. When an organic solvent is passed through the columns, the lipophilic compounds are extracted and collected in the eluate, while the water-soluble compounds are retained in the columns. This principle was soon adapted for the extraction of hormones \( (7-9) \). I have applied this technique to serum estradiol determination.

Materials and Methods

For the extraction I use 5-mL \( (9 \text{ mm, i.d.}) \) Brunswick syringes (Sherwood, West Sussex, U.K.), from which the pistons have been removed. In the bottom of the syringe, place a 9-mm phase-separating filter \( (1 \text{ PS}; \text{Whatman, Maidstone, U.K.}) \). Onto this filter, pour 2 mL \( (0.45 \text{ g}) \) of kieselguhr of grainy structure \( (\text{Extrelut}\text{®}; \text{Merck, Darmstadt, G.F.R.}) \), then settle it with the use of a vortex-type mixer. On top of the kieselguhr layer, place a 9-mm diameter filter paper. To produce a suitable elution rate, stick a cannula \( (\text{Yale}\text{®} \text{21G} 1/2; \text{Becton Dickinson, Rutherford, NJ 07070}) \) on the effluent cone.

Apply 100 \( \mu \text{L} \) of sample or standard to the column, then 650 \( \mu \text{L} \) of distilled water, and allow it to drain into the matrix to saturate it with water phase. Elute with two 5-mL portions of diethyl ether \( (\text{p.A. grade}; \text{Merck}) \), collecting the eluates in 5-mL \( (11 \times 75 \text{ mm}) \) glass tubes. Evaporate the first eluate under a stream of air on a water bath set at 40 °C, and then elute the column with the second 5-mL portion of the ether, which is collected in the same tube. Again evaporate the ether, dissolve the residue in buffer, and perform the radioimmunoassay.

Use of a special rack \( (\text{Figure 1}) \) facilitates the procedure. One can handle 40 columns at a time, placing them in the upper part of the rack. The collecting tubes are placed in the lower part and, after elution, the upper part is removed and replaced by the air supply system, consisting of stainless-steel tubes \( (10 \text{ mm, i.d.}) \) connected to an external source of compressed air. Fit each steel tube with needles \( (0.8 \text{ mm, i.d.}) \), for outlet of the air to the surface of the collecting tubes \( (\text{Figure 2}) \). In this way, seven standards, two controls, and 11 samples, in duplicate, can be extracted simultaneously.

For radioimmunoassay of estradiol I used commercially available reagents \( (\text{EstrK}; \text{CIS, Sorin, Italy}) \). After separation of free and bound \( [\text{H}] \text{estradiol with dextran-coated charcoal,} \)
0.5 mL of the supernate was mixed with 8 mL of scintillation cocktail (Aqualuma®, Lumac Systems A.G., Basel, Switzerland) and radioactivity was counted with a liquid scintillation counter (Beckman LS-150; Beckman Instruments, Fullerton, CA 92634), with automatic quench correction.

Results

The 0.45 g of kieselguhr used in the columns bound 0.75 mL of ether, in addition to all of the water phase. To evaluate the extraction yield, I applied 5 nCi of [2,4,6,7-3H]17β-estradiol (the same quantity used per test in the EstrK kit) to the columns, in 0.75 mL of distilled water. After elution, performed in one or two steps, the analytical recovery of the radioactivity was as shown in Table 1. Nearly quantitative elution (97.7%) was obtained with use of two 5-mL portions of ether, the total effluent volume being 9.25 mL. In all further experiments, I used this extraction procedure.

The dextran-coated charcoal bound >95% of the tritiated estradiol, when no antibody was present. This degree of binding was the same, when I extracted the serum on the column and assayed the residue after evaporation without addition of antibody.

After column extraction the antibody used in the kit bound an average of 40% of the added tritiated estradiol.

Figure 3 shows a log-linear representation of the mean and SD for 20 standard curves. Counts for the other standards are expressed relative to counts for the zero standard (B/B0). A volume of 0.1 mL was assayed, so the absolute range covered by the curve is 0.028–0.88 pmol. Two control sera (Ortho RIA Controls III and IV; Ortho Diagnostic Systems, Raritan, NJ 08869) were divided into aliquots, stored at −20 °C, and later assayed on different occasions. Control III, assayed 15 times during six weeks, gave a mean of 0.84 nmol/L and a CV of 10.8%. Control IV, assayed 10 times during a month, gave a mean of 2.27 nmol/L and a CV of 8.4%. When 26 patients' sera (range 0.25–5.25 nmol/L) were assayed with use of the extraction procedure described for the kit and with use of the present column procedure, mean values were 1.83 nmol/L for the new (y) assay, and 1.64 nmol/L for the usual (x) procedure, and the linear regression equation was y = 1.12x − 0.006 nmol/L (r = 0.98).

Discussion

In radioimmunoassays, many tubes are usually processed at the same time. Exact timing is very critical, and exposure of many samples with use of a mechanical shaker or a rotating mixer is laborious and introduces serious problems: it is difficult to stopper tubes containing volatile organic solvents, emulsions may form, a centrifugation step may be necessary to separate the phases, and the organic phase must be transferred from the extraction tubes with the risk of loss. These problems have been overcome in the proposed extraction method. The principle is generally applicable for extraction of drugs and hormones, and the rack we constructed greatly facilitates the simultaneous handling of many samples.

The extraction yield seems to be almost quantitative, which may explain the about 12% higher results I obtained with the new procedure as compared with usual extraction. The extract does not contain constituents from the columns that damage the antibody or the charcoal binding. About 40% of the total activity is bound by antibody, when no unlabeled estradiol is present; the package insert for the kit prescribes 30–50% binding. The interassay CVs from this study, 8–10%, compare favorably with those (15–18%) given in the package insert. The standard curve is reasonable consistent from assay to assay, and results by the two extraction procedures agreed well. The price of one column—including syringe, cannula, filters and kieselguhr material—is about $0.20. Samples can easily be loaded manually or by use of an automatic pipettor/diluter, and one technician can apply, elute, and evaporate the solvent from 40 tubes in about 30 min.

I thank Mr. Carl B. Larsen, Technical Department, Centralasylehus i Naestved, for constructing the extraction and evaporation rack.

References

p 460: The decimal in “4.0” is not clearly obvious in the body of Figure 4.

p 462: Column one, third paragraph, line 24: “50 pg” should read “50 ng.”

p 580: Authors’ corrections for this paper were received too late. The following corrections are needed. Abstract, last sentence: substitute “volatile profiles” for “these volatiles”.

p 787: Boehringer Mannheim Corp. no longer supports the Award mentioned in column one. Boehringer Mannheim Diagnostics (formerly Hycel) supports the Award mentioned in column two.

p 884: The authors of the abstract on this page (Ash) and that on p 885 (Ferone) are interchanged.

p 1041: The abstract numbered 084 here was printed again elsewhere. Abstract 084 should read:

Comparison of Du Pont 2ac and Dow Methods for the Determination of High Density Lipoprotein Cholesterol (HDLCH).

We compared the determination of total cholesterol (TCH) and HDLCH in sera by the Du Pont 2ac and Dow methods, using specimens from 114 patients. Measurement of HDLCH by these two methods is based on separation of non-HDLCH by precipitation, followed by enzymatic determination of HDLCH in the supernate. The ratio (R) TCH to HDLCH was calculated for each specimen. The following linear regression equations were obtained using the Statistical Analysis System:

\[
\begin{align*}
\text{TCH:} & \quad \text{2ac} = 1.20 \text{ Dow} - 6 \text{ mg/dL} \\
\text{HDLCH:} & \quad \text{2ac} = 1.10 \text{ Dow} - 0.4 \text{ mg/dL} \\
\text{R:} & \quad \text{2ac} = 0.96 \text{ Dow} - 0.0
\end{align*}
\]

A comparison of the results using the paired-sample Student’s t-test showed that the 2ac result for TCH and HDLCH was significantly \(p < 0.0001\) greater than the Dow result. No significant difference was found between the calculated ratios.

Three control sera were analyzed in duplicate during this three-month period to estimate precision. The results are the following (mg/dL ± SD):

<table>
<thead>
<tr>
<th>Control</th>
<th>2ac</th>
<th>Dow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.9 ± 2.0</td>
<td>22.8 ± 2.1</td>
</tr>
<tr>
<td>2</td>
<td>38.1 ± 3.8</td>
<td>37.9 ± 3.7</td>
</tr>
<tr>
<td>3</td>
<td>35.6 ± 2.5</td>
<td>32.2 ± 2.5</td>
</tr>
</tbody>
</table>

Billirubin (up to 10.7 mg/dL), hemoglobin (up to 200 mg/dL), and slight lipemic (triglycerides up to 500 mg/dL) did not alter the HDLCH result with the 2ac system. Thus there is a systematic bias between the 2ac and Dow methods for determination of TCH and HDLCH, but they compare favorably with respect to precision.

p 1045: The author of abstract no. 105 is V. B. Kamble (Norwalk Hospital, Norwalk, CT 06856).

p 1190: Under Materials and Methods, line 7: change “μmol” to “mmol” in both mentions.

p 1389: Column two, line 3: “Table 1” should read “Table 2.” Table 2, not originally supplied, should have read as follows:

Table 2. Statistical Comparison of Urinary Protein Studies in Three Histopathologically Distinct Groups of Children with Nephrotic Syndrome

<table>
<thead>
<tr>
<th>Protein study</th>
<th>H-test</th>
<th>FGS vs. MPGN</th>
<th>MPGN vs. MC vs. MPGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary excretion</td>
<td>Albumin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>α2-Macroglobulin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urinary clearance</td>
<td>Albumin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>α2-Macroglobulin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Selective index</td>
<td>clearance of IgG/transferrin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+ indicates significance; - indicates no significance at a “p” level of 0.05.

b H-test is derived by the Kruskal–Wallis method (9). This test compares all of the patients. c U-test is derived by the Mann–Whitney method (9). This test compares the groups for differences.

p 1472: Column one, fifth full paragraph down: the word “restricted” should read “unrestricted.”