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Estimation of True Values in Radioimmunoassays

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We tested the accuracy of radioimmunoassays for two hormones, progesterone and estradiol, in relation to the use of two different antisera in each assay and to the degree of purification from plasma before the assay was done. Each analyte was assayed either in a diethyl ether extract, in a zone eluted from a Sephadex LH-20 chromatographic column, or in fractions of a chromatographic zone (from Sephadex LH-20 or Celite) that passed a test of "radiochemical purity." Statistically indistinguishable results were obtained in the assay of radiochemically pure fractions of both analytes, irrespective of the antiserum used. In addition, one of the antisera from each hormone gave equivalent results in the radioimmunoassay of ether extracts, even with no preceding chromatography. We demonstrated in this way that results obtained with use of highly specific antisera may, after a single chromatography, but even without any chromatographic purification, be as nearly accurate and as closely reflect the true value as those obtained in the assay of radiochemically pure hormones, an assay that has a character of a reference method (as defined by the IFCC).

Additional Keyphrases: *steroids · reference methods · analytical error · sample treatment · column chromatography on Sephadex, Celite*

A radioimmunoassay that is analytically valid gives results that are accurate, i.e., they estimate the true value, within the limits of experimental error (*1, 2*). To demonstrate the validity of a series of steroid radioimmunoassays, we previously used a "test of radiochemical purity"[4] (*3–8*) to ascertain whether or not chromatographic purification of a biological material yielded a radiochemically pure compound (i.e., a compound devoid of any admixture cross reacting with the antiserum used). If the purity was demonstrated, then all radioimmunoassays performed with the same chromatographic pre-purification and the same antiserum were considered valid. Assays not involving chromatography but providing the same results were also considered valid.

We now wish to show that the "test of radiochemical purity" can be modified to measure the analyte directly in the radiochemically pure fractions, and thus it can become a reference method for testing the validity of assays performed with or without preceding chromatography.

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[4] The term "radiochemical purity" in this context means a degree of purity that is characterized by a constant specific activity (statistically indistinguishable ratios of radioactivity to immunoreactive mass, e.g., dpm/fmol) in individual fractions of a chromatographic zone (*1*).

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Materials and Methods

Reagents. [1,2,6,7-^{3}H]Progesterone (110 kCi/mol) and [2,4,6,7-^{3}H]estradiol (105 kCi/mol) were from Amersham International, Amersham, U.K. All other reagents were of analytical grade.

Plasma was pooled from 3-mL blood samples collected into heparinized tubes from each of 12 normally menstruating women daily during one cycle. The plasma, obtained by centrifugation, was pooled with regard to the cycle day. Thus we formed five pools (nos. 1–5) of differing progesterone and estradiol contents and kept them at −20 °C until assayed. A sixth plasma pool (no. 6) was obtained by combining samples taken predominantly during the follicular phase of the menstrual cycles of 26 women; this pool was also stored at −20 °C.

For chromatography, we used columns packed with Sephadex LH-20 (Pharmacia, Uppsala, Sweden) or Celite (Manville Products, Denver, CO 80217).

The following antisera were obtained from the Matched Reagents Programme, World Health Organization, Geneva, Switzerland: for progesterone, batch K0789 (polyclonal, denoted here PA) and batch K8736 (monoclonal, PB); for estradiol, batch K1583 (polyclonal, EA) and batch A-EST (polyclonal, EB). The calibration materials (progesterone and estradiol standards) were obtained from the same source.

Radioimmunoassay. Radioimmunoassays of progesterone and estradiol involving overnight incubation and charcoal separation were performed according to the WHO Method Manual (*9*).

Extraction and chromatography. Various aliquots of plasma (from 50 μL to 1 mL, depending on the experiment and the steroid content of the plasma) were extracted by vortex-mixing with a 10- to 20-fold volume of diethyl ether, except for the assays of progesterone in plasma pools nos. 1–5, for which we used a 20-fold volume of *n*-hexane. The solvent was evaporated, and the residue was dissolved either in 0.5 mL of assay buffer (phosphate buffer, pH 7.4, 0.1 mol/L, containing 8.8 g of NaCl, 0.1 g of thimerosal, and 1.0 g of gelatin per liter; *9*) or in an appropriate solvent for chromatography.

For the chromatography on Sephadex, 24 × 0.8 (i.d.) cm glass columns were purchased from Isolab Inc., Akron, OH 44321. The eluent was equivolume toluene/methanol for progesterone and toluene/methanol (8/2 by vol) for estradiol. The samples were applied in 0.5 mL of the appropriate solvent mixture and eluted at ambient temperature. Progesterone was eluted between milliliters 4 and 6, estradiol between milliliters 8 and 11.

The chromatography on Celite was carried out in disposable glass columns at 18 °C as described earlier (*10*). For progesterone, the columns were packed with Celite (1 kg/L) suspended in propylene glycol and washed with isooctane (10 mL). The sample was added in 1 mL of isooctane, and progesterone was eluted with the same solvent, in milliliters 2 through 5. For estradiol, Celite (2 kg/L), suspended in ethylene glycol, was used in packing the column. The columns were washed with isooctane (10 mL), the sample

was added in isooctane (1 mL), and the column was further washed with isooctane/toluene (8/2 by vol; 5 mL) and isooctane/ethyl acetate (85/15 by vol; 6 mL). Estradiol was obtained in the last 3 mL of the last-named eluent.

Test of radiochemical purity. Labeled progesterone (90 000 dpm) or labeled estradiol (75 000 dpm) was added to 7.5 and 12 mL, respectively, of plasma pool no. 6.

The plasma was divided into 0.5-mL portions for progesterone and 1-mL portions for estradiol. Each portion was extracted with 5 mL of diethyl ether, once for progesterone and twice for estradiol. The ether phase was evaporated and the residue was dissolved in the corresponding chromatographic solvent (0.5 mL for Sephadex and 1 mL for Celite chromatography). Each solution was chromatographed separately and 0.5-mL fractions were collected.

Three sets of columns were run, each consisting of five columns for progesterone and four columns for estradiol. In each set, the same fractions from the different columns were pooled to obtain values for mass that were within the range of the standard curve. This pooling of fractions was in some instances guided by the measurements for radioactivity. In other instances, when the elution pattern was very reproducible, the fractions were pooled according to the fraction number.

One set of columns represented one replicate; each pooled fraction thus had three replicates. The pooled fractions were evaporated and reconstituted with 1 mL of assay buffer: 0.5 mL was used for the radioimmunoassay and 0.4 mL for the measurement of radioactivity (analytical recovery).

Calculations. We calculated the specific activity (dpm/fmol) for each pooled fraction. A one-way analysis of variance (ANOVA; *11*) was computed for the specific activities in all pooled triplicate fractions of a chromatography. Only those fractions were included for which >2% of the applied sample was present (if less, the variation in specific activities was very high, owing to low counts and concentrations). If the ANOVA indicated a significant difference between the means of replicate (n = 3) measurements of specific activities at the 95% confidence level, we calculated another ANOVA, in which the fraction having the lowest mean of specific activity was excluded. This process was repeated (if necessary) until the ANOVA did not indicate any difference between means. If only two means remained, the difference was tested by Student's *t*-test. The radiochemical purity test was considered invalid if the difference was significant by the *t*-test.

In all pooled fractions, the concentrations were computed from the radioimmunoassay data and recoveries, with corrections (*12*) for the variable amounts of tracer in individual fractions. Only the concentrations in the fractions with constant (statistically indistinguishable) specific activities were considered to be estimates of true value, and these were averaged for each chromatography and antiserum.

To test differences between means of concentrations measured with individual antisera and procedures, we used an ANOVA.

Results

In the assay of progesterone, antiserum PA consistently yielded results significantly higher than those obtained after chromatography (Figure 1). Antiserum EA similarly affected results in the estradiol assay (Figure 2). For the remaining two antisera (PB and EB), the results were

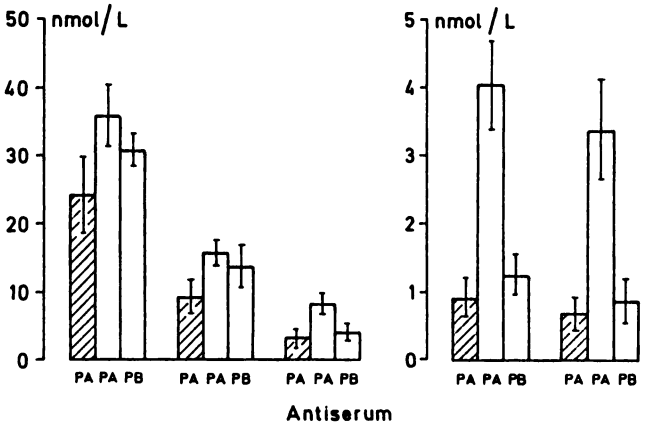

Fig. 1. Means and 95% confidence limits of progesterone concentrations in five plasma pools

The radioimmunoassays were done with use of antisera PA and PB, after Sephadex chromatography *(hatched columns)* or without chromatography *(empty columns)*

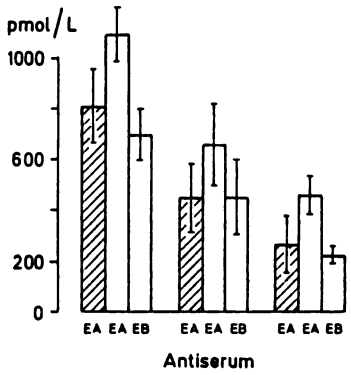

Fig. 2. Means and 95% confidence limits of estradiol concentrations in three plasma pools, as measured with antisera EA and EB

Symbols as in Fig. 1

indistinguishable from those obtained after chromatography.

Using the same antisera and plasma pool no. 6, we carried out tests of radiochemical purity. Figure 3 shows results of a typical test of radiochemical purity for progesterone assayed after chromatography on Sephadex with use of antiserum PB. The specific activity was constant (statistically indistinguishable) in two fractions (5.0 and 5.5 mL), as assessed by *t*-test. We calculated the concentration (2.05 nmol/L) as the mean of the values found for the two fractions.

Table 1 gives the number of fractions with constant specific activity for each hormone, type of chromatography, and antiserum. In the assay of progesterone, chromatography on Celite yielded a greater number of radiochemically pure fractions (three and five for antisera PA and PB, respectively) than did chromatography on Sephadex (two for each antiserum). In the estradiol assay, chromatography on Sephadex gave a higher number of pure fractions (five each for antisera EA and EB) than did chromatography on Celite (one and three, respectively).

The concentrations calculated in the radiochemically pure fractions were compared by ANOVA with those obtained without chromatography. At the 95% confidence level, progesterone values were indistinguishable for all assays except for that without chromatography and with

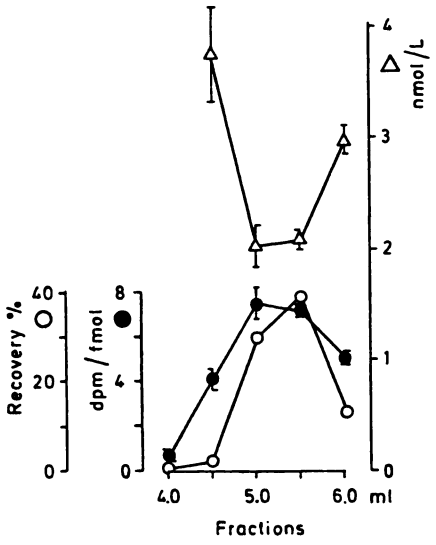

Fig. 3. An example of the test of radiochemical purity for progesterone in pool no. 6, determined with antiserum PB and Sephadex chromatography run in triplicate

Specific activities (●) in fractions 5.0 and 5.5 mL were statistically indistinguishable, and progesterone in these two fractions was considered radiochemically pure. Therefore, the concentrations in these two fractions (△) were taken as estimates of the true value. Their average was 2.05 nmol/L (*see* Table 1). If calculated without correction for the presence of tracer, this value was 1.52 nmol/L, a 26% negative bias

use of antiserum PA, which resulted in a significantly higher value. For the estradiol assay, only the procedure involving antiserum EA without chromatography gave significantly higher results than the other procedures.

Discussion

The first approximation of the true value can often be obtained by an immunoassay preceded by a chromatographic purification of the biological material, especially when comparisons of various extraction techniques (*4*) or antisera (*13, 14*) are involved. This contention is supported by the present assays of progesterone and estradiol in several plasma pools. As supported by the other data of this study, the results obtained after chromatography and the equivalent results obtained with two of the four antisera tested without chromatography are likely to closely reflect the true values.

A reference analytical method such as gas–liquid chromatography/mass spectrometry is usually used to establish a true value properly in assays of steroids and other compounds, including drugs (*6*). However, there are practical problems in many instances. In addition, the reference method has its own experimental errors (*6, 15*).

We demonstrated previously (*6*) that the reference method may be replaced in establishing the true value by a test of "radiochemical purity." This test can identify the radiochemically pure zone in a chromatography. Implicitly, if the same chromatography is later used as a purification step before the assay proper, true values should be obtained in this assay. However, this approach has the disadvantage that the elution pattern may undergo changes, albeit small, in repeated chromatographies and that a portion of cross-reacting impurities may be co-measured, thus biasing the true value.

This disadvantage is avoided in the present paper by estimating the true value during the process of testing radiochemical purity, by determining the concentrations in those chromatographic fractions in which the radiochemical purity had been demonstrated. This direct assay of pure fractions thus assumes the character of a reference method.

The purity of the compound can be taken for granted if the specific activity (e.g., in dpm/fmol) is constant in several chromatographic fractions (i.e., when the peaks of radioactivity and mass coincide). In previous papers (*3–7*), we examined the constancy of the specific activities statistically by regression analysis. We accepted the constancy when the slope of specific activities was indistinguishable from zero and when linearity of the values was not contradicted by the data.

In the present investigation, we chose ANOVA as an equivalent, but simpler, statistical procedure. We assumed the constancy of specific activities (and thus radiochemical purity) if the calculated *F*-value for the difference in means did not exceed the tabulated statistic at the 95% confidence level. It goes without saying that the higher the number of replicates for each pooled fraction, the better is the estimate of the experimental error and the higher the statistical sensitivity of the test.

This statistical approach makes it possible to decrease the number of fractions with a constant specific activity to a minimum of two, in which case Student's *t*-test can be used. This low number of fractions with constant activities is obviously associated with a considerable degree of uncertainty, and the procedure is permissible only in association with other observations (as presented here) or as a preliminary observation.

The number of chromatographic fractions having a constant specific activity depends on two factors. The first is the specificity of the antiserum. With a less-specific antiserum there is more chance that cross-reacting impurities will decrease the specific activity before and (or) after the peak of the pure analyte (see Table 1 for a higher number

Table 1. Average Values of Progesterone and Estradiol in a Female Plasma Pool

	Progesterone antiserum						Estradiol antiserum					
	PA			PB			EA			EB		
Chromatographic separation	n[a]	Mean, nmol/L[b]	CV, %	n	Mean, nmol/L	CV, %	n	Mean, pmol/L	CV, %	n	Mean, pmol/L	CV, %
None	2	4.14[c]	14.2	2	2.25	5.0	3	305[c]	16.4	3	180	13.2
Sephadex LH-20	2	1.95	7.2	2	2.05	3.5	5	196	6.2	5	204	8.9
Celite	3	1.73	3.0	5	1.66	14.1	1	201	0	3	177	11.3

[a] For assays without chromatography, n=number of repeated measurements (each being a mean of four replicates); for chromatographic separations, n=number of pooled fractions containing radiochemically pure compound (each pooled fraction was measured in three replicates).

[b] Differences between means were tested with use of a one-way analysis of variance, followed by the calculation of appropriate contrasts.

[c] Significantly higher (at the 95% confidence level) than the other means obtained with the same antiserum.

of pure fractions with the more-specific antisera PB and EB in the Celite chromatography). The second is the volume of the fraction in relation to the broadness of peak; e.g., 0.5-mL fractions were too large in the Sephadex chromatography of progesterone or in the Celite chromatography of estradiol.

Interestingly, the estimates of true value that we obtained on the basis of only two fractions were statistically indistinguishable from the observations based on three to five fractions, and the estimates were independent of the type of chromatography. Moreover, in one case (Table 1; antiserum EA, Celite) one single fraction with the highest specific activity (the preceding fractions contained cross-reacting impurities) gave a good estimate of true value, despite the fact that the test of radiochemical purity was formally invalid.

After averaging all five statistically indistinguishable observations per steroid, the estimates of the true value (with 95% confidence limits) were 1.9 nmol/L (1.5–2.3 nmol/L) for progesterone and 190 pmol/L (170–220 pmol/L) for estradiol in that plasma pool. The confidence limits could be made narrower in a dedicated experiment with more replications.

The true-values estimation shown here is to be considered an example of the procedure. This estimation can be carried out with all samples or pools of blood (plasma or serum), urine, and other body fluids. The volume required depends on the concentration of the analyte. To ensure reliable radioimmunoassay measurements in the pooled fractions, more columns are needed per pooled fraction with a low-concentration material, and vice versa.

In the calculation of concentrations it is essential to make an appropriate correction (*12*) for the tracer present in variable amounts in individual fractions. If such a correction is not made, underestimation in the high-recovery fractions (containing most of the tracer) can be as high as 25% (*see* Figure 3). Thus the estimate of true value could be grossly erroneous if the corrections were not carried out.

The estimation of true values by means of a radioimmunoassay is described above for two steroid hormones. Clearly, however, the same procedure should be applicable to all other compounds for which a reliable calibration material and a tritiated tracer are available.

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