A Strategy for Radioimmunoassay of Plasma Progesterone with Use of a Homologous-Site $^{125}$I-Labeled Radioligand

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In this sensitive, specific $^{125}$I radioimmunoassay for plasma progesterone, an identical glucuronide bridge is used in the immunogen and radioligand. This apparently provides a system in which the bridge is poorly recognized and thereby avoids the excessively high affinity of the tracer for the antiserum and the consequent poor sensitivity that commonly is found in other homologous-bridge situations. The glucuronide bridge is potentially useful for attachment of other labeling probes.

Additional Keyphrases: statistics · steroids · immunochemistry

The advantages that might be gained by replacing $^3$H with $^{125}$I as the emitting nuclide in the radioimmunoassay of small molecules have been enumerated frequently in recent years. Many such assays have been described, particularly for the estimation of drugs (1), but among the major gonadal steroids, where the high workload renders particularly valuable the attributes of $^{125}$I-based assays—especially work-simplification, shorter counting times, and decreased costs—there is still a heavy reliance on $^3$H systems. Two vital features of assay performance that are often diminished by the use of radioiodinated tracers, namely sensitivity and specificity, underlie this conservatism. To incorporate $^{125}$I into a steroid one usually must prepare a conjugate of the steroid and an iodinatable aromatic species (generally histamine, tyramine, or tyrosine methyl ester), and this is most conveniently done by using the same bridge structure (e.g., hemisuccinate or carboxymethylxime) as appears in the steroid–protein conjugate that is used as the immunogen (2). Because at least a proportion of the antibodies elicited by the immunogen recognize the bridge as well as the steroid, such radioligands generally have a higher affinity for the antibody than does the analyte, and consequently the assay is likely to be less sensitive than the corresponding system where a $^{3}$H-labeled radioligand, identical in structure with the analyte, is used. One means by which sensitivity may be restored is to vary the structure of the bridge with the aim of reducing the affinity of the radioligand for the antibody. This approach has been shown to provide a sensitive standard curve (3), but it is entirely empirical, and for a given antiserum it might be necessary to test several different bridges in the radioligand before finding one suitable. Other workers have suggested that subtle variation of the bridge can be beneficial (4).

An alternative strategy is a heterologous-site assay, in which the bridge in the immunogen and in the tracer is attached to different positions on the steroid. Again, such systems are unpredictable: in some cases the tracer fails to bind to the antiserum while other combinations generate sensitive standard curves (5), but these are generally accompanied by a deterioration in the specificity of the assay because two areas of the steroid molecule are obscured.

Despite these difficulties, both homologous- (5, 6) and heterologous-site (7) $^{125}$I-based assays for progesterone have been described, but Cameron et al. (5, 6) note that among groups of antisera that yielded acceptable assays with a $^3$H-labeled ligand, only exceptional antisera were suitable for use with a $^{125}$I-labeled tracer. Clearly, a major obstacle to the wider use of iodinated tracers is related to the nature of the bridging structure used to functionalize the steroid, and we have been concerned to seek potentially generalized solutions to this problem.

Kellie and co-workers (8, 9) have reported that when antisera were raised against steroid glucuronides, where the glucuronide carboxyl group was used to link the steroid to a carrier protein for preparation of the immunogen, the corresponding free steroid showed a high degree of cross reactivity. We reasoned that such antisera must be relatively blind to the bridge and that the attachment of an iodine-bearing species to the glucuronide might permit the development of a sensitive assay. We have used a glucuronide prepared from 11α-hydroxyprogesterone, and the results obtained form the subject of this report.

Materials and Methods

General Chemical and Radioimmunoassay Materials

5α-Pregnane-3,20-dione was from Steraloids, Croydon, Surrey, and pregn-4-en-20α-ol-3-one was from the M.R.C. Steroid Reference Collection, Westfield College, London. All other steroids, tyramine, N-hydroxysuccinimide, and bovine serum albumin were from Sigma, U.K. Na$^{125}$I (IMS.30) and [1,2,6,7-$^{3}$H$_4$]progesterone were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-a-D-glucopyranuronate was prepared by the published procedure (10) from methyl 1α,2,3,4-tetra-O-acetyl-d-glucopyranuronate, which was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Sephadex LH20 and Sepharose 4B were from Pharmacia Ltd., London. Cyanogen bromide and dicyclohexylcarbodiimide were from Aldrich, Gillingham, Dorset, U.K. Preparative thin-layer chromatography plates were prepared from Merck silica gel HPl$^{254}$.

All other common solvents and reagents were from B.D.H. Chemicals Ltd. and were used as received, except for petroleum ether, 40–60°C, low in aromatic hydrocarbons (cat. no. 10184), which was stirred overnight with one-tenth its volume of concentrated sulfuric acid, then for a further 6 h with a fresh portion of acid, decanted, and washed with water and saturated sodium bicarbonate, dried over anhydrous magnesium sulfate, and distilled.

New Zealand White rabbits were from Ranch Rabbits Ltd. and Freund’s adjuvant from Difco. Donkey anti-rabbit serum was from the Scottish Antibody Production Unit and was coupled to Sepharose 4B as described by Cuatracaas (11). Assay diluent was 0.05 mol/L phosphate, pH 7.5, containing 1 g of gelatin per liter, and assay tubes were Corning 12 x 75 mm Pyrex culture tubes. Progesterone standards were prepared in ethanol solution in doubling concentrations over the range 0.40–51.20 μg/L (20–2560 pg in the assay tube) and stored at 4°C.
Plasma samples were from clinical specimens received by the Immunoassay Section, Department of Clinical Chemistry, Royal Infirmary of Edinburgh, and had been previously estimated by that laboratory in an established radioimmunoassay involving use of a tritiated radioligand (12).

Beta-counting was done in a Searle “Isocap” ligand scintillation counter with 4 g/L Butyl-PBD in toluene as the scintillation fluid. Gamma-counting was in a “Wallac” autogamma counter.

Preparation of Immunogen and 125I-Radioligand

Methyl 23,4-Tri-O-acetyl-3,20-dioxopregn-4-en-11α-yl-β-D-glucopyranosiduronate (I) was prepared according to Nambara et al. (13). Freshly precipitated and dried cadmium carbonate (0.90 g) was added to a stirred solution of 11α-hydroxyprogesterone (0.80 g) in anhydrous toluene (480 mL) and the solution was heated in an oil bath to distill about 10 mL of solvent. A solution of 1.50 g of methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate in 8 mL of toluene was added, and the mixture was concentrated by slow distillation during 5 h. Further portions of the bromoester (2.40 g) in toluene (25 mL) and cadmium carbonate (0.30 g) were added and slow distillation was continued for 1 h. The mixture was cooled and filtered, the filter cake was washed with toluene and the combined filtrates were evaporated under reduced pressure. A solution of the oily residue in chloroform was chromatographed on a column of 60–120 mesh silica gel. Elution with chloroform/ethyl acetate (4/1 by vol) gave a crude fraction, which contained the steroid conjugate. Portions (50–60 mg) of this material were purified by preparative layer chromatography on 20 x 20 x 1 mm silica gel plates, which were developed twice in chloroform/ethyl acetate (7/3 by vol). The required band (Rf 0.65–0.7) was visible under ultraviolet light, and the product was recovered by elution with chloroform. The combined eluates were evaporated, triturated with a little diethyl ether, and allowed to stand at 4 °C overnight. The solid was filtered and recrystallized from aqueous methanol to afford the product as colorless needles (0.13 g), m.p. 195–8 °C, suitable for further use. Two more crystallizations gave an analytical sample, m.p. 207–9 °C (Found: C, 61.85; H, 7.2. C23H24O12 requires C, 61.4; H, 7.3%). λmax (E1% 1 cm) 241 nm (ε 17,250); λmax 1755, 1740, 1705, 1665 cm⁻¹; 1H n.m.r. spectrum (CDCl3) 5.566 (1H, s, H-4), 4.80–5.30 (3H, m, CHOAc), 4.64 (1H, d, pyranose C-1-H, J = 7 Hz), 3.86–4.20 (2H, m, H-11 and pyranose C-5-H), 3.70 (3H, s, OCH3), 2.11 (3H, s, H-21), 2.00 (6H, s, —OOCCH3), 1.98 (3H, s, —OOCCH3), 1.27 (3H, s, H-19) and 0.67 (3H, s, H-18).

3,20-Dioxopregn-4-en-11α-yl-β-D-glucopyranosiduronide acid (II) was prepared by a modification of the method of Mattox et al. (14). The methyl ester triacetate (I) (128 mg) was dissolved in 0.2 mol/L methanolic sodium hydroxide (11 mL) and kept at room temperature for 20 min, 11 mL of water was added, and the solution was kept for 30 min, then acidified with acetic acid/methanol (1/2 by vol) and evaporated under reduced pressure. The residue was dissolved in 15 mL of water, brought to pH 2 with 1 mol/L sulfuric acid, and applied to a prewashed (14) column of Amberlite XAD-2 (50 g). The column was washed with 300 mL of water, then eluted with 450 mL of absolute ethanol. The ethanolic solution was evaporated under reduced pressure and the residue redissolved in 30 mL of water and lyophilized to leave the acid (II), trivially called 11α-progesterone glucuronide, as a colorless, amorphous solid (97 mg).

When the acidification steps were omitted, the sodium salt of the glucuronide was obtained.

11α-Progesterone glucuronide–bovine serum albumin conjugate was prepared by a modified Erlenmeyer procedure (15). The sodium salt of 11α-progesterone glucuronide (66 mg) dissolved in anhydrous dimethyl formamide (1.27 mL) was cooled to 10 °C, mixed with tri-n-butylamine (28 μL) and isobutyl chloroformate (13.5 μL) and kept at 10 °C for 0.5 h, then added to an ice-cold solution of 1.50 mg of bovine serum albumin in 3.97 mL of water, 0.15 mL of 1 mol/L sodium hydroxide, and 2.65 mL of dimethylformamide. The solution was kept in an ice-bath for 3.5 h, dialyzed overnight against 50 mmol/L phosphate, pH 7.5, and then chromatographed on 250 mL of Sephadex G50 in the same buffer. The pooled protein represented by the peak was dialyzed overnight vs distilled water and lyophilized. Analysis by ultraviolet spectroscopy and Lowry protein estimation (16) gave a coupling ratio of 10.8 progesterone residues per protein molecule.

Antisera were raised in New Zealand White rabbits as described previously (17), with the modification that three of the six animals were given reduced doses (100 μg) of immunogen. Antiserum dilution curves and standard curves were obtained with [3H]progesterone tracer and liquid-phase donkey antirabbit serum separation as described (17). 11α-Progesterone glucuronide–tyramine conjugate was prepared by a modification of the method of Beckett et al. (18) (see Figure 1). A solution of 76 mg of 11α-progesterone glucuronide and 19 mg of N-hydroxysuccinimide in 3.6 mL of dry tetrahydrofuran was treated with a solution of 34 mg of dicyclohexylcarbodiimide in 0.6 mL of dry tetrahydrofuran. The solution was kept at room temperature for 3 h and a solution of 24 mg of benzoic acid in 0.3 mL of tetrahydrofuran was added to destroy unreacted carbodiimide. After 1 h the precipitated dicyclohexylurea was filtered off and washed with ethyl acetate, and the combined filtrates were evaporated under reduced pressure. The residue was dissolved in a solution of 23 mg of tyramine in 1.8 mL of dry dimethylformamide, kept at room temperature for 3 h, and the solvent removed under reduced pressure. The residual gum was mixed with a saturated solution of sodium bicarbonate in 3 mL of brine and extracted with three 3-mL portions of ethyl acetate. Analytical thin-layer chromatography on silica gel HF254 in ethyl acetate/methanol (3/1 by vol) revealed three major phenolic components (identified by spraying with Folin–Ciocalteau reagent) with RF 0.90 (N-benzyloxytyramine), 0.1–0.2 (tyramine), and 0.65. The RF 0.65 spot showed strong fluorescence quenching when the plate was viewed under ultraviolet light and was assigned as the expected conjugate.

The ethyl acetate extract was evaporated under reduced pressure, redissolved in ethyl acetate/methanol (9/1 by vol) and chromatographed on an 18-g column of Kieselgel H (Type 60; Merck) in the same solvent. Fractions (0.5 mL) were collected, monitored by thin-layer chromatography, and those that contained only the pure conjugate were pooled and evaporated. The residue was washed with diethyl ether and dried to give 40 mg of the conjugate (III) as a colorless,
amorphous powder, which was dissolved in methanol and stored at 4 °C. The 1H n.m.r. spectrum (methanol-d₄) showed δ 7.04 (2H, d, Ar-H, J = 8 Hz). 6.71 (2H, d, Ar-H), 5.71 (1H, s, H-4), 4.53 (1H, d, pyranose C1-H, J = 8 Hz), 2.16 (3H, s, —COCH₃), 1.27 (3H, s, H-19) and 0.70 (3H, s, H-18).

Iodinated radioligand. An aliquot of the ethanolic solution containing 380 ng of the tyramine conjugate was evaporated in a stream of nitrogen and the residue redissolved in 10 µL of ethyl acetate, then treated with Na[125]I (1 mCi) and Chloramine T (10 µL of 5 mg/mL solution in 0.25 mol/L phosphate, pH 7.5) and mixed vigorously for 30 s. Cysteine hydrochloride (10 µL of 10 mg/mL in 0.05 mol/L phosphate, pH 7.5) was added to reduce unused [125]I, followed by potassium iodide (200 µL of 1 mg/mL solution in 50 mmol/L phosphate, pH 7.5). The solution was extracted with 300 µL of ethyl acetate and the extract was diluted with 200 µL of methanol, applied to a 14 × 1 cm column of Sephadex LH 20, and eluted with ethyl acetate/methanol (3/2 by vol). One-milliliter fractions were collected and fractions 8–10 were pooled and stored at 4 °C. The estimated specific activity of the tracer was 500 kCi/mol, calculated by comparison of the self-displacing ability of increasing amounts of the radioligand with a standard curve of the non-iodinated conjugate (19). Before use, an aliquot of the solution was evaporated under nitrogen and reconstituted in assay diluent.

Radioimmunoassay Procedures

Monitoring progesterone extraction from plasma. An ethanolic solution of [3H]progesterone was dispensed into glass tubes (75 × 17 mm) to give 5000 counts/min per tube, and the solvent was removed under nitrogen. Aliquots (50 µL) of progesterone-containing plasma were added and allowed to equilibrate for 15 min. Aqueous ethanol (3/2 by vol; 50 µL) was added and the contents of the tubes were vortex-mixed and incubated at room temperature for 20 min. Purified petroleum ether (2 mL) was added and the contents of the tubes were mixed on a multi-vortex shaker for 5 min. The tubes were immersed in an alcohol–solid CO₂ bath, the organic layer was decanted into assay tubes, and the solvent was evaporated by heating for 20 min at 55–60 °C under a stream of nitrogen. Assay buffer (200 µL) was added to the dried residues and the contents of the tubes were vortex-mixed for 5 min, allowed to stand for 15 min, and then diluted to 2 mL with buffer. The tube contents were decanted into scintillation vials, 10 mL of scintillation fluid was added, and the vials were capped, shaken vigorously for 2 min, and their radioactivity was measured.

Assay. Ethanic standard solutions of progesterone were dispensed into assay tubes and the solvent was removed under a stream of nitrogen. Samples were prepared as dried residues in assay tubes as described above, except that no [3H]progesterone was added before the extraction. Assay buffer (200 µL) was added to the contents of all tubes, which were vortex-mixed for 5 min and allowed to stand at room temperature for 15 min. Radioligand (13 fmol; about 6000 counts/min when first prepared; 50 µL) and antiserum at 6000-fold dilution (50 µL) were added, the tubes’ contents were vortex-mixed briefly and allowed to stand at room temperature for 2 h. Sephacore-coupled donkey anti-rabbit serum, 20-fold diluted (100 µL) was added, the tubes were agitated for 0.5 h, and separated by two cycles of the previously described sucrose layer technique (20, 21). Then assay diluent (0.5 mL) was added rapidly and the solution allowed to settle for 5 min. Sucrose (1 mL of 100 g/L solution in assay diluent) was layered beneath the incubation mixtures, with a 16-channel peristaltic pump, and after 20 min all but the lower portion (about 0.3
mL) of the tube contents was aspirated to waste. A further 0.5 mL of assay diluent was added, the cycle was repeated as above, and the remaining (antibody-bound) fraction was counted with the gamma counter. We processed the data by computer, using a modified four-parameter logistic fit (22) to construct the dose–response curve; results were corrected for extraction recovery.

Results

Antisera. Three of the six rabbits produced antisera of acceptable titre, and Figure 2 shows the standard curves obtained with tritiated and 125I-labeled radioisotopes from one of these antisera (R6/10). The two remaining antisera gave insensitive standard curves with both radioisotopes, the 125I-labeled tracer affording standard curves that were between three- and 10-fold less sensitive (as measured at 50% tracer displacement) than those obtained with the tritiated ligand. No consistent effect attributable to the different immunogen dose levels was observed.

Iodinated radioisotope. The observed specific activity is about 25% of the theoretically attainable value for a homogeneous mono-125I-labeled compound. Attempts to improve the specific activity by reduction of the mass of conjugate taken for iodination were vitiated by a concomitant reduction in the percentage incorporation.

Specificity. Major cross reactions at 50% displacement (23) of the antiseraum R6/10 used with the 125I-labeled radioisotopes were from 5α-pregnane-3,20-dione (10%), 20α-hydroxy-pregn-4-ene-3-one (2.8%), and 17α-hydroxyprogesterone (1.2%).

Sensitivity and working range. The formal sensitivity of the assay system, expressed as the mass of analyte that gives a decrease in percentage binding of tracer of 2.5 × SD of percentage tracer bound in the absence of unlabeled progesterone (24), was 8 pg per tube, corresponding to 0.51 nmol/L in serum. It has previously been noted (17) that the precision of determinations at the normal detection limit is poor, and in practice we have accepted only those results that fell between cutoff levels of 20 to 80% depression of B0, giving a working range for the assay of 32–2200 pg per tube, i.e., 2–140 nmol/L in serum.

Extraction recovery. The percentage recoveries by petroleum ether extraction, as described above, of [3H]progesterone added to low-, medium-, and high-concentration pools were (mean ± SD) 92.6 ± 1.3, 92.3 ± 1.2, and 94.9 ± 2.1, respectively. Because of the observed consistency of the results, analytical recoveries for individual samples were not measured. Instead an overall recovery correction, determined for the three pools at the beginning of each assay, was applied.

Precision and accuracy. Table 1 shows the within-assay component of the variation of duplicate means for 93 unknown samples assayed among six consecutive assays, calculated from the formula

\[
\text{Within-assay CV} = \frac{1}{n} \sum_{i=1}^{n} \frac{d_i}{4s_i^2} \sqrt{n}
\]

where \( \bar{x} \) is the mean result for the ith specimen and \( d_i \) is the difference between the two duplicate values. Each unknown sample was assayed once (in duplicate), and no result was excluded from the statistical analysis.

Progesterone was added to plasma from normal men, to establish three pools of low, medium, and high concentration, which were assayed in five consecutive assays. The results for accuracy of recovery and between-assay variation appear in Table 2. No allowance was made for endogenous progesterone, which averages 0.65 nmol/L (17) and accounts for the observed trend of excess recovery from the low- and medium-concentration pools.

| Table 1. Within-Replicate Precision of Duplicate Mean Estimations of Unknown Samples over Various Concentrations |
|---|---|---|---|
| Conc. range, nmol/L | No. pairs | CV, % |
| All samples | 93 | 7.4 |
| 2–10 | 22 | 9.9 |
| 10–40 | 55 | 6.2 |
| 40–90 | 16 | 7.7 |

| Table 2. Analytical Recovery and Interassay Variation of Progesterone Added to Normal Plasma |
|---|---|---|---|
| Progesterone added | Progesterone measured | Recovery, % | Inter-assay CV, % |
| nmol/L | | |
| 5.1 | 5.9 | 115 | 13.5 |
| 12.7 | 14.3 | 113 | 11.5 |
| 41.0 | 41.8 | 102 | 8.1 |

* Uncorrected for endogenous progesterone.

Discussion

The principal object of the study was to examine the utility of a glucuronide bridge as a weakly antigenic subunit, whose common presence in an immunogen and radioiodinated tracer would be poorly recognized by an antibody. Although none of the antibodies obtained in the various antisera were entirely indifferent to the presence of the bridge structure, the relatively small rightward shifts of standard curves (compared with those obtained with tritiated progesterone as radioisotopic tracer) suggest that the use of immunogen/tracer pairs which incorporate a homologous-site glucuronide bridge is likely to facilitate the establishment of sensitive and specific iodine-125-based radioimmunoassays for haptens.

The single-extraction procedure used is a minor and more reproducible revision of the method described earlier (17) and is so consistent and nearly quantitative as to obviate either the need for a double extraction or the individual monitoring of recoveries.

Use of a liquid-phase primary incubation followed by solid-phase second antibody combined a short equilibration time with the benefits to precision and convenience of a solid-phase separation system. No evidence of drift was observed; the assay is therefore well suited to handling large sample batches. Sensitivity is more than sufficient to fulfill the clinical requirement of a progesterone assay, i.e., the monitoring of luteal function. Estimation of progesterone concentrations during the follicular phase or in male plasma would necessitate extraction of larger plasma volumes.

In conclusion, the use of the glucuronide bridge has enabled
us to develop a specific progesterone radioimmunoassay in which a \(^{125}\text{I}\)-labeled tracer is used, which has sensitivity much improved over that previously reported (26) with hemisuccinate-bridged compounds (Figure 3). The glucuronide appears to offer a rational means to attach external labels to haptons without incurring the need to select an exceptional antiserum from a large group, and we intend to investigate other models to establish more firmly the generality of this approach.

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References
Statistical Appendix

G. Raab

When a set of n specimens is assayed by each of two methods we have n pairs of results \((x_i, y_i)\), \(i = 1, \ldots, n\), in which \(x\) and \(y\) are both subject to random errors of measurement. Thus neither the regression of \(x\) on \(y\) or that of \(y\) on \(x\) will give an unbiased estimate of the underlying relationship \(Y = \alpha + \beta X\) between the two assay methods. When \(\sigma_x^2\) and \(\sigma_y^2\) are the two measurement error variances and their ratio

\[
\lambda = \frac{\sigma_y^2}{\sigma_x^2}
\]

is assumed to be known, we can obtain an unbiased estimate \(\hat{\beta}\) of the slope from the quadratic equation:

\[
\hat{\beta}^2 S_{XX} + \hat{\beta} (\lambda S_{XX} - S_{YY}) - \lambda S_{XY} = 0
\]

where

\[
S_{XX} = \sum_i (x_i - \bar{x})^2, \quad S_{YY} = \sum_i (y_i - \bar{y})^2
\]

\[
S_{XY} = \sum_i (x_i - \bar{x})(y_i - \bar{y})
\]

\[
\bar{x} = \sum_i x_i / n, \quad \bar{y} = \sum_i y_i / n
\]

An unbiased estimate \(\hat{\alpha}\) of \(\alpha\) can be obtained by taking the line with slope \(\hat{\beta}\) which passes through the point \((\bar{x}, \bar{y})\). When \(\lambda = 1\), this is equivalent to fitting the line by minimizing the sum of the squares of the perpendicular distances from the observations to the fitted line. An unbiased estimate of \(\sigma^2\) is given by:

\[
\hat{\sigma}^2 = \frac{\lambda}{(n - 2) (\lambda + \hat{\beta}^2)} (S_{YY} - 2\hat{\beta} S_{XY} + \hat{\beta}^2 S_{XX}).
\]

When \(\lambda = 1\), this estimates \(\sigma_x^2\) and \(\sigma_y^2\) and measures the average squared perpendicular distances from the observations to the fitted line. The method is discussed by Mandel (1).

A \(p\%\) confidence interval (2, 3) for the slope \(\hat{\beta}\) can be obtained from the formula

\[
\frac{(S_{YY} - \lambda S_{XX}) + \sqrt{(S_{YY} - \lambda S_{XX})^2 + 4\lambda(S_{XY} - Q)}}{2 S_{XY} \pm 2\sqrt{Q}}
\]

where \(Q = \frac{t_{p,n-2}^2}{t_{p,n-2}^2} (S_{XX} S_{YY} - S_{XY})/(n - 2)\), and \(t_{p,n-2}\) is the two-sided \(p\%\) point of the \(t\) distribution with \(n - 2\) degrees of freedom.

As \(\sigma^2\) is approximately (for large \(n\)) distributed as \(\chi^2_{n-2}\), we can also obtain confidence intervals for our variance estimates.

The present case is in fact slightly more complex, because it is the coefficients of variation of the two assays that are approximately constant and equal, rather than their standard deviations. The above analysis was applied with the means and sums of squares replaced by the equivalent weighted means and sums of squares, where the weight of the \(i\)th pair of observations was taken as

\[
\frac{100}{(x_i + y_i/2)}
\]

The variance estimates then become estimates of the squared percent coefficients of variation. The theory is then only approximately satisfied, because we are using the average observations for the two methods, rather than the “true” values of \(X\) or \(Y\) in the weights. However, the coefficients of variation of the two assays are sufficiently small and \(\alpha\) and \(\beta\) close enough to 0 and 1, respectively, to make this approximation unimportant.

References