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A COMPUTERIZED SEMI-AUTOMATED RADIOIMMUNOASSAY FOR PLASMA TESTOSTERONE

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SUMMARY

A new computer program has been developed for the treatment of data from steroid radioimmunoassays. It uses an iterative technique that solves some 600 quadratic equations to compute the binding parameters for two independent, saturable binding agents from the standard curve data. The accuracy of curve fitting was such that all points on the standard curve were within one standard deviation of perfect fit, and no systematic bias was introduced into the results. Because the binding parameters were independent of the labelled ligand concentration, the methodology could be simplified, partially automated, and made more accurate and precise. The method was rapid, and 112 male plasma samples and 14 quality controls could be analysed (duplicate extracts on 40 μl aliquots with duplicate assays on each extract) by one person in one and a half days. The coefficient of variation between duplicate extracts of a reference plasma sample containing about 1000 ng testosterone/100 ml was 3.1% and the variation between assays on this sample conducted over a 3-month period was 3.8%.

INTRODUCTION

The radioimmunoassay of steroids has opened new areas for research in endocrinology, but the particular method of conducting assays must be based on the special requirements of each laboratory. Our own need was for a testosterone assay capable of analysing the large number of samples generated in a long-term study on the relationship between plasma testosterone levels and behavior in male rhesus monkeys. Optimum accuracy and precision, stringent quality control, and low unit cost were essential. Two factors have led to the design of an assay with improved performance, (i) the production of antisera sufficiently specific to avoid the need for chromatography, and (ii) the availability of very precise and accurate automatic pipettes.

To assess the inevitable losses that occur in any assay during sample preparation, either an external [1, 2] or internal standard must be used. Internal standards increase reliability but interfere with the assay of the sample. These difficulties are minimized in systems employing [^{125}I]-steroid derivatives [3]. Internal standards are either added in small amounts [4–11] so as to interfere minimally with the assay (which requires a second addition of labelled steroid), or in large amounts [12–15] so as to serve as both recovery tracer and labelled ligand. The second method is simpler, lends itself to automation, and allows a more rapid and accurate estimation of recovery. However, because the amount of labelled steroid in the assay tubes is variable, computational methods, such as the log-logit transformation [16], that rely on its constancy should not be used, and published methods have resorted to tedious, graphical solutions. To avoid this, we have developed a theoretically based, iterative technique suitable for use on a minicomputer.

EXPERIMENTAL

Reagents and solutions

Water was freshly glass distilled and ethyl ether (Fisher Scientific Co. "Anhydrous grade") was double distilled immediately before use. Testosterone was obtained from Steraloids Inc., and testosterone [1,2,6,7-^{3}H(N)] (95 Ci/mmol) (New England Nuclear) was purified by chromatography on paper in the Bush 'A' system [17]. The antiserum was kindly supplied by Dr. Philip Rowe and had been raised against testosterone-3-carboxymethyl-oxime-BSA. Cross reactivities of this antiserum have been described elsewhere [10]. The antiserum was used at a final dilution of 1:24000. All other reagents were of analytical grade. Phosphate buffered NaCl in 0.1% gelatin (PBSM-gel) and dextran coated charcoal in PBSM-gel were prepared as described elsewhere [10, 18]. A testosterone stock solution (2.00 mg/ 100 ml ethanol) was prepared and working solutions were made up as dilutions of this. Ethanolic solutions were stored at −20°C and aqueous solutions at 2°C. Immediately before each assay, two tritiated testosterone solutions were prepared: for the standard curve (solution A) 1.6×10^6 d.p.m. [^{3}H]-testosterone blown down with nitrogen and redissolved in PBSM-gel (3 ml), and for the diluent solution (solution B) 9×10^6 d.p.m. [^{3}H]-testosterone blown into 150 ml water and mixed thoroughly. Solution A (100 μl) and solution B (1 ml) were checked for similar levels of radioactivity.

Sample preparation and assay

Plasma samples, stored at −20°C, were allowed to thaw at 2°C overnight and were then centrifuged at 2000 *g* for 10 min to help remove fibrin. Six standards containing 0, 200, 500, 1000, 1500 and 2000 ng/100 ml of testosterone in water, and a reference male rhesus monkey plasma pool containing about 1000 ng/100 ml testosterone were prepared: these were assayed in parallel with the plasma samples. Automatic sampling of plasma was found to be unreliable because of the blockage of the pipette tip with fibrin. An automatic pipette (Micromedic Systems Inc.) was therefore used manually to dispense 20 or 40 μl aliquots of the plasma sample or quality control in duplicate together with 1 ml [^{3}H]-testosterone solution B into glass stoppered test tubes standing in racks from the automatic pipetting station (APS, Micromedic Systems Inc.). The diluted sample was allowed to stand at room temperature for at least 20 min to allow the [^{3}H]-tracer to equilibrate with the steroid binding proteins of the plasma. Freshly distilled ether (3 ml) was dispensed into each tube which was stoppered, shaken vigorously, left for 10 min and then shaken again. The tubes, still in the APS racks, were then placed in a freezer at −20°C to freeze the aqueous layer. Ether extracts were decanted from all tubes in each rack into 12 × 75 mm tubes in matching APS racks in a single operation. The ether was blown off with N_2 and 200 μl PBSM-gel was added to each dried extract. The tubes were then agitated in an orbital shaker for 10 min to ensure that the testosterone was dissolved. For the direct (unextracted) standard curve the zero standard containing no unlabelled testosterone was prepared as 1:1 dilution of the [^{3}H]-testosterone solution A with PBSM-gel. The 200 pg/50 μl direct standard was made up similarly by diluting solution A with a testosterone solution containing 200 pg/25 μl of PBSM-gel. The intermediate points (containing 25, 50, 100 and 150 pg testosterone/50 μl) were prepared by making appropriate dilutions of the zero and 200 pg standards. The standards were arranged in an APS rack with a seventh tube (NC = no charcoal treatment) containing the same mixture as the zero standard. This served to give an estimate of the amount of [^{3}H]-testosterone added to the direct standards. Beyond this step the samples and direct standards received identical treatment. Fifty μl aliquots were sampled by the APS and dispensed with 150 μl of antiserum into 12 × 75 mm disposable culture tubes in duplicate. The direct standards and the NC tube were sampled in quadruplicate. Finally, 50 μl aliquots of the samples and quality controls were dispensed with 1 ml water into minivials for estimation of procedural losses. Assay tubes were then covered and placed in the cold room overnight at 2°C. To separate free from bound testosterone, 1 ml dextran-coated charcoal suspension was added to each tube (except the NC tubes which received 1 ml PBSM-gel). The tubes were shaken vigorously, left for 10 min and then centrifuged at 2000 *g* for 10 min. Eight racks containing 112 tubes were treated simultaneously. This resulted in a maximum difference of 2 min in the period of exposure to charcoal which caused less than 2% variation in the estimate of counts bound. Supernatants in each rack were decanted into minivials in a single operation, and the loss of material during the decanting was 5 ± 1%. Toluene-Triton X-100 scintillator cocktail (3.5 ml) was dispensed into the vials which were shaken and left in the dark for some time before counting. Radioactivity estimations (Packard Model 3385) were continued to 10,000 counts for each vial. The punched tape output from the scintillation counter was fed into a minicomputer (Digital Corporation PDP8/E with 16K core), and the data were stored on magnetic tape and disc.

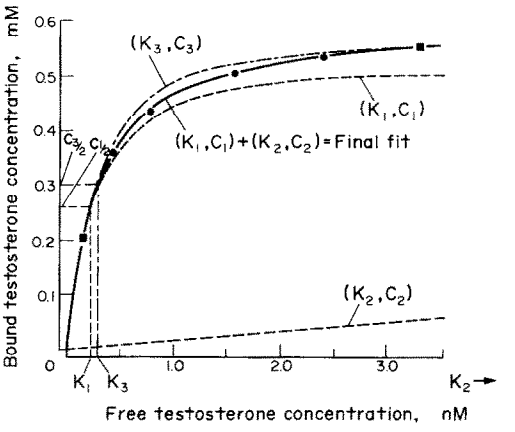

Fig. 1. Fitting the theoretical binding curve for two independent binding agents (K_1, C_1 and K_2, C_2) to the experimental points of a standard curve. The curve for a single binding agent (K_3, C_3) drawn through the defined points (■) at 0 and 200 pg of unlabelled testosterone is consistently above the intermediate points (●) at 25, 50, 100 and 150 pg unlabelled testosterone. At half saturation (bound testosterone = $C_3/2$), the value of K_3 is indicated by the free testosterone concentration (labelled + unlabelled), and must be greater than K_1. The sum of the curves for K_1, C_1 and K_2, C_2 provides an excellent fit to the experimental points. For clarity, the individual binding curves have been altered, and the actual values were: $K_1 = 2.76 \times 10^{-10}$ M, $C_1 = 5.79 \times 10^{-10}$ M, $K_2 = 1.73 \times 10^{-6}$ M, $C_2 = 1.07 \times 10^{-8}$ M, $K_3 = 2.92 \times 10^{-10}$ M, $C_3 = 6.04 \times 10^{-10}$ M.

Treatment of data

A computerized iterative technique using a theoretically based equation was used to generate binding parameters that were independent of the amount of [^{3}H]-testosterone present. The dissociation constant (K_3) and the corresponding concentration (C_3) in a mass action equation for a single binding agent generally gave an inaccurate fit to the experimental points (Fig. 1). To improve upon this, we have used a 4-parameter equation for the case of 2 independent, saturable binding agents. This was a quadratic of the form:

$$T_F^2 \cdot Y + T_F \cdot [(K_1 + K_2)Y - C_1 - C_2] + K_1K_2Y - K_1C_2 - K_2C_1 = 0$$

where T_F = free testosterone concentration, K_1 and K_2 are the dissociation constants for the two binding agents and C_1 and C_2 are their respective concentrations. Y is the bound/free testosterone ratio determined experimentally. A first approximation of K_1 and C_1 was made by calculating K_3 and C_3 in a mass action equation for a single binding agent (Fig. 1). To fit the same points in an equation for two binding agents, one dissociation constant (K_1) must be lower than K_3 and the other (K_2) must be higher. Thus the computer searches between K_3 and zero for the best value of K_1 (Fig. 1). The initial guess for K_1 was $K_3/2$, and for C_1 was $C_3/2$. For the second binding agent C_2 and K_2 were calculated as:

$$C_2 = \frac{T'_F - T''_F}{\dfrac{K_1 + T'_F}{Y'(K_1 + T'_F) - C_1} - \dfrac{K_1 + T''_F}{Y''(K_1 + T''_F) - C_1}}$$

and

$$K_2 = \frac{C_2(K_1 + T'_F)}{Y'(K_1 + T'_F) - C_1}$$

where primed and double primed symbols refer to the experimental values at the extreme points of the standard curve (0 and 200 pg unlabelled steroid). The existence of a unique minimum in the error is necessary for the method. The program proceeds by comparing the error (see below) for the current best fit point with the new error from a point first below and then (unless a new best fit was encountered) above it. The excursion from the current best fit is progressively halved until either a satisfactorily small error is obtained or a built-in counter causes the program to exit. A similar routine was used to optimize C_1 for each value of K_1.

To calculate the total error, the value at each intermediate point was divided by (i) the mass of unlabelled testosterone to correct for pipetting error and (ii) the experimental variance (4 assays/point) to allow for the non-uniformity of variance. The best fit parameters were used to calculate the unknown testosterone concentration (X) in each sample from the positive root (T_F) of the quadratic equation and the [^{3}H]-testosterone concentration (T^*) by:

$$X = \frac{T^*}{T^*_F}(T_F - T^*_F)$$

where T^*_F is the concentration of free [^{3}H]-testosterone. The value of T^* was determined from the counts in the recovery vial multiplied by the mean recovery for the charcoal treatment (0.95) since the recovery aliquot was pipetted and the bound fraction was decanted. Results were corrected for procedural losses but not for the value of the water blank. Copies of this OS/8 Fortran II program are available from the authors.

RESULTS

Evaluation of parameter fitting programme

Figure 2 shows the error of the fit to the four intermediate points (25, 50, 100, and 150 pg) in a 0–200 pg standard curve plotted as a function of K_1. The concentration (C_1) of the first binding agent has been optimized for each point on the error curve and K_2 and C_2 are fixed once K_1 and C_1 have been determined. There is a unique minimum in the error which justifies this parameter fitting method. The values of K_1 determined by successive approximations during the parameter fitting are superimposed on the error curve; the final value lies very close to the minimum error point. The error term used in the figure is the root of the sum of deviations squared divided by the masses squared. Similarly, for C_1 the error curve at fixed values of K_1 has a unique minimum which generally lies close to a discontinuity where C_2 and/or K_2 increase rapidly and then become negative.

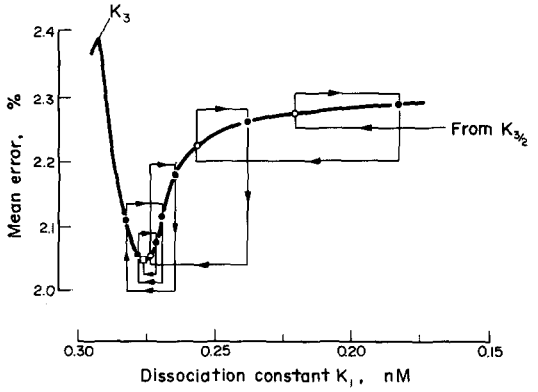

Fig. 2. Error curve for K_1 at optimized values of C_1 in a mass action equation for the binding curve of two independent binding agents fitted to the experimental points. The maximum error occurred where $K_1 = K_3$. Successive approximations made by the computer in searching for a best-fit value for K_1 are superimposed on the curve. When a point with a lower error (○) was encountered, a new search was made about that value; points with a greater error (●) were discarded. The final best fit value (□) was used to calculate results. The percent mean error was calculated as:

$$\sqrt{\sum_{i=2}^{5}\left(\frac{x_i - y_i}{0.01x_i}\right)^2}\Big/4$$

where $x_{2\to5}$ are the actual values of the intermediate points of the standard curve and $y_{2\to5}$ are the computed values.

Exactness of curve fitting

Figure 3 shows the ratios of fitted values to actual values for each intermediate point in the direct standard curves of seven routine large-scale assays. The points are scattered above and below the horizontal line at the perfect fit value (1.0). The 0 and 200 pg points in each standard curve were defined automatically by the programme and exactly fit the final curve. Of the 42 points in the 7 standard curves, two were more than 5% out and 31 were within 2% of actual values. All points were within one standard deviation of perfect fit, and this is shown in Fig. 3 where the vertical standard deviation bars intersect the horizontal line at 1.0.

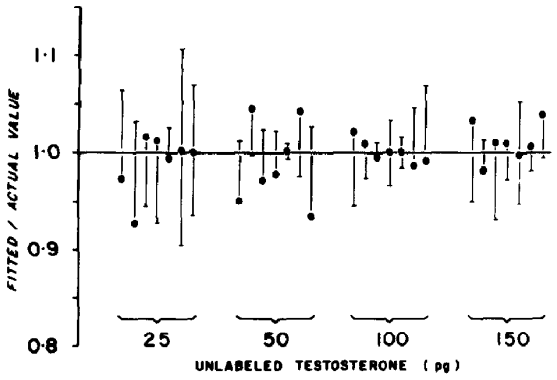

Fig. 3. Accuracy with which the computer routine fitted a curve to the intermediate points in 7 standard curves. The fitted/actual ratios were calculated from the means (●) ± 1 standard deviation (vertical bars) of the counts bound in quadruplicate assays. Points were scattered above and below the perfect fit value of 1.0 and showed no consistent bias. All the fitted values were within one standard deviation of perfect fit.

Independence of binding parameters and [^{3}H]-testosterone concentrations

To test the capacity of the computer program to determine binding parameters that were independent of the amount of [^{3}H]-testosterone present, 5 direct standard curves were each assayed in quadruplicate (0, 25, 50, 100, 150 and 200 pg). The amount of [^{3}H]-testosterone added to each tube ranged from 21.51 pg for curve 1 down to 4.38 pg for curve 5. Table 1 (B values) shows the per cent of testosterone bound in each standard curve. Predictably, the per cent bound increased with decreasing [^{3}H]-testosterone added. However, this effect does not influence the binding parameters. Curve 2, 19.65 pg [^{3}H]-testosterone, was used to determine these ($K_1 = 2.63 \times 10^{-10}$ M, $C_1 = 5.81 \times 10^{-10}$ M, $K_2 = 3.91 \times 10^{-6}$ M, $C_2 = 1.04 \times 10^{-8}$ M). Table 1 (T values) gives the mean mass of unlabelled testosterone calculated at each point in the 5 standard curves using these parameters. Errors in the curve fitting occurred in curve 5 which has less than a quarter of the [^{3}H]-testosterone concentration of curve 2. There was, however, a trend, evident at 150–200 pg for the binding parameters to predict high values where [^{3}H]-testosterone concentrations were low. This trend was too small to produce errors in the assays where recoveries, and therefore [^{3}H]-testosterone concentrations, vary by $< 10\%$.

Accuracy and precision of the assay

To assess the reliability of the method two trial assays were performed on aqueous testosterone solutions prepared in the same way as the quality controls in routine assays. In Trial 1, 8 solutions each of the following testosterone concentrations were made up: 0, 400, 1000, 2000, 3000 and 4000 ng/100 ml. In addition, assays were conducted on plasma obtained from a castrated male rhesus monkey bearing a subcutaneous Silastic implant of testosterone. Duplicate aliquots (20 μl) of each solution were extracted with 5 ml ether (112 extractions), and treated as described above for the standard assay method. A similar procedure was followed in Trial 2, except that 2 series of processed standards were prepared containing 0, 200, 500, 1000, 1500 and 2000 ng/100 ml (28 extractions), and half of each extract was discarded to test the method's ability to cope with low recoveries. For Trial 1, the mean blank (i.e. the apparent mass of testosterone in each assay tube of the zero processed standard) was 6.49 ± 3.51 pg (± S.D.) (N = 32), and the mean recovery was 91.2 ± 6.4% (± S.D.) (N = 112). For Trial 2, with deliberately reduced recoveries, the mean blank was 0.92 ± 1.78 pg (N = 8) and the mean recovery was 46.2 ± 0.8% (N = 28). The results of these trials are shown in Table 2. Coeff. var. 1 gives the variation between the means of the duplicates on the two extracts of each sample; it reflects the precision of sample preparation

Table 1. The dependence of the per cent bound parameter (B) on [^{3}H]-testosterone concentration and the lack of its effect on the pg of unlabelled testosterone (T) calculated with the binding parameters of curve number 2

Unlabelled testosterone (pg)		[^{3}H]-testosterone added (pg) 21.5	19.7	15.0	10.6	4.4
0	B	60.9%	60.2%	63.6%	64.8%	71.4%
	T	−3.5	0.0	−1.3	2.0	1.1
25	B	47.7%	47.3%	48.9%	52.7%	57.1%
	T	23.1	26.2	28.0	25.8	29.8
50	B	37.1%	37.6%	40.8%	42.7%	45.2%
	T	51.2	51.9	48.0	49.0	57.4
100	B	26.9%	27.3%	28.1%	29.3%	30.5%
	T	97.1	97.2	99.2	100.7	115.2
150	B	20.9%	20.7%	21.7%	22.1%	23.9%
	T	146.6	152.6	150.6	157.8	181.5
200	B	17.3%	17.4%	17.8%	18.0%	19.0%
	T	197.4	200.0	204.6	211.1	275.2
		1	2	3	4	5
				Standard curve number		

Table 2. Accuracy and precision of method in two trial assays of testosterone standard solutions and a plasma sample

Testosterone concentration (ng/100 ml)	Assay means (ng/100 ml)	Variation between duplicate extracts (Coeff. var. 1)	Variation between duplicate assays on each extract (Coeff. var. 2)	Variation between testosterone standards (Coeff. var. 3)
Trial 1. 20 μl sample—8 duplicate extractions				
0	139.4	30.5	62.5	33.0
400	517.6	5.5	12.2	13.7
1000	1037.7	1.9	5.1	6.1
2000	1973.8	1.7	3.9	5.6
3000	3027.1	0.9	3.8	2.3
4000	3995.0	1.6	5.3	4.6
Plasma sample	1040.1	1.0	4.8	4.2
Trial 2. 20 μl sample—deliberately reduced recoveries				
0	39.6	63.3	200.7	90.7
200	300.5	19.8	44.4	5.5
500	660.6	9.5	19.7	9.2
1000	1148.9	4.2	8.9	3.4
1500	1698.1	4.5	8.3	2.4
2000	2364.0	3.8	8.4	3.3
Plasma sample	1203.0	3.5	11.7	7.9

and recovery estimation. Coeff. var. 2 gives the variation between the two assays on each extract; it reflects the precision of the method after sample preparation, including charcoal treatment and counting. Coeff. var. 3 gives the variation between means (duplicate assays on duplicate extracts) for 8 replicate assays (8 in Trial 1) of the same testosterone concentration; this reflects systematic changes in technical error over the time-course of the assay. The accuracy of the assay is given by comparing the mean values with the actual testosterone concentrations.

Quality controls comprising 5 testosterone standards (200–2000 ng/100 ml), a water blank, and a sample from a reference pool of male rhesus monkey plasma (about 1000 ng/100 ml) were routinely assayed with each batch of unknowns. Table 3 shows the accuracy and precision of these quality controls in seven successive assays conducted over a 3-month period. The mean blank was 6.2 ± 3.4 pg ($\pm$ S.D.) ($N = 38$) and the mean recovery for the quality controls and samples was $91.1 \pm 2.5\%$ ($N = 784$). The assay means were all slightly higher than the actual concentration in the standards, and were more precise at the higher concentrations. Results in Tables 2 and 3 show that the interassay variation (different standard curves and different antiserum solutions) was almost the same as the variation between parallel samples in the same assay.

Table 3. Accuracy and precision of quality controls in seven routine assays

Testosterone concentration (ng/100 ml)	Assay means (ng/100 ml)	Variation between duplicate extracts (%)	Variation between assays (%)
0	56.9	111.7	57.4
200	257.5	18.9	14.2
500	571.9	6.7	5.2
1000	1111.5	3.7	6.9
1500	1621.3	3.5	4.3
2000	2197.0	4.6	6.9
Plasma pool	955.5	3.1	3.8

DISCUSSION

The computerized curve-fitting program satisfied three criteria for use in a radioimmunoassay: (i) it was accurate, (ii) it did not introduce a consistent bias into the results, and (iii) it generated binding parameters that were independent of the range of [^{3}H]-testosterone concentrations in the assay. Furthermore, the well-defined minimum error (Fig. 2) indicated that the properties of the antiserum were better described by two saturable binding agents than by one. However, since we used charcoal to separate free from bound testosterone, the binding parameters calculated would have been affected by the perturbation of the equilibrium that charcoal causes [19]. The new method differs from other computerized techniques that generate binding parameters [20–22] in: (i) the simplification of the program by the forced fitting of the extreme points of the standard curve, (ii) the generation of its own first guess, (iii) its suitability for use in processing radioimmunoassay data, and (iv) its compatability with minicomputers. The program was designed to fit an equation as closely as possible to the standard curve, and this ensured the accuracy of the assay. The program was not intended to calculate the real binding parameters of the system. This requires a more complex statistical method [22, 23], a wider range of ligand concentrations, and a more conservative method of measureing the concentration of bound ligand. Simplification

of the statistics was made possible by the precision of the automatic pipettes. The programme may not be suitable where large variances are encountered because the implicit assumption of homogeneity of variance in the fitted variable (mass) may no longer hold. However, since no assumptions were made about the state of saturation of the binding agents, the computation method may be used in assays operated far from saturation where such curve fitting routines as the log-logit transformation [16] introduce a consistent bias into the results. Because the iterative program solves some 600 quadratic equations in each curve fitting routine, a computer is essential. However, the graphical solution of Furuyama *et al.*[12] would be applicable, though tedious and less accurate.

This assay avoids steps that might multiply errors; in fact, some are partially self-correcting. For example, an error in the removal of a recovery aliquot would be balanced by an opposite effect on the assayed value. Thus, the coefficients of variation (1000 ng testosterone/100 ml) for (i) duplicate extracts (1.9%) and, (ii) duplicate assays on the same extract (5.1%) (Tables 1 and 2) are highly satisfactory. It was evident that the major remaining sources of error arose during the charcoal treatment and during the estimation of radioactivity (both about 1%).

The following were important in determining the accuracy of the assay: (i) the exact preparation of the standard curve, (ii) the accuracy of the computerized curve fitting, and (iii) the avoidance of contamination with testosterone binding inhibitors. Inaccuracies in (i) and (ii) would have caused errors in both directions, but all the actual errors were positive, and therefore probably attributable to contaminants. Competitive inhibitors of low affinity (relative to testosterone) would elevate most the readings of the lower standards, whereas non-competitive inhibitors, or competitive inhibitors of high affinity, would increase the assay values equally throughout the whole range of concentrations. Since both effects occurred (Tables 1 and 2), a subtraction of the blank value could not be justified.

A competent technician working alone was easily able to conduct assays on 112 samples and 14 quality controls in one and a half days. The counting time was about three days and the total computer time was 30–45 min. The cost per sample for materials (excluding antiserum) was about 50 cents-U.S. in a 50 sample assay. Lack of uniformity in reporting reliability criteria makes it difficult to make direct comparisons with other published methods. However, we can find none that out-performs the new method on precision and accuracy. Moreover, it uses a smaller plasma volume, allows more samples to be assayed in shorter time and, as far as can be ascertained, at lower cost. The method has been comprehensively validated and offers clear advantages to laboratories with minicomputer facilities.

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