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RAPID RADIOIMMUNOASSAY OF OESTRADIOL-17β USING COMPUTER CALCULATION

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SUMMARY

A new rapid solid phase radioimmunoassay for the determination of oestradiol-17β (E_2) in plasma which uses computer calculation is described. An approximation to a linear dose-response relationship obtained from plots of ratios of unbound (U)/bound (B) radioactive steriod against dose enables a formulae based in terms of U and B to be devised for the calculation of E_2 in plasma. Radioimmunoassay B and U c.p.m. data was fed into a computer programmed to process the formulae, but assessment could be made using a simple calculator. Assay time was saved because the method obviates the need for lengthy recovery count, preparation of full standard curves, and with the use of specific antisera, chromatographic separation is not necessary. The new solid phase radioimmunoassay possessed good precision, and gave results which were in excellent agreement with those obtained by the normal technique. Derivation of the formulae is given, and the advantages of the method discussed. In the case of direct radioimmunoassay only B or U values need be known. The mathematical approach has general application for any type of radioimmunoassay or radioligand assay which can provide a linear dose-response curve passing through the origin.

INTRODUCTION

DESPITE the apparent simplicity of present day steroid radioimmunoassay techniques, all the methods involve time consuming operations preventing rapid estimation. Since direct radioimmunological determination of steroids is not yet possible[1], all methods involve prior extraction of plasma with organic solvent. In order to account for procedural losses during extraction, a radioactive recovery estimate is usually made prior to actual radioimmunoassay. Unfortunately like that of many other hormones the peripheral plasma concentration of oestradiol-17β (E_2) is extremely low, and this means that even with the use of very high specific activity tetra-tritiated E_2 (circa 100 Ci/m mol) only a very small amount can be added, otherwise this exogenous E_2 mass would be greater than the endogenous mass, preventing accurate assay. Again with the normal method of recovery assessment, the amount of radioactivity added must be kept very low otherwise the amount carried over in the aliquot for radioimmunoassay proper, being variable in amount, could seriously interfere with the assay. Due to this the aliquot taken after extraction for recovery assessment invariably contains low c.p.m with the consequence that for a reasonable, accurate determination of this radioactivity long counting times are necessary with concomitant increase in assay time. Further increase in assay time is caused by the use of standard curves which must be set up for each individual group of assays. This invariably involves estimating some 5 or 6 standards in duplicate along with the assay.

Previous efforts to devise transforms which yield precise linear dose response relationships assessable for calculation of assay results have led to the logit transform $\log_e y/(1-y)$ against $\log_e$ (dose) where y is the response variable % bound divided by % bound at zero dose[2], and its mathematical equivalent $\log_e$

($U/B - U_0/B_0$) against $\log_e$ (dose) where B_0 equals the bound and U_0 equals the unbound at zero dose[3]. These transforms although varyingly successful in yielding linear dose response curves are relatively complicated with the result that the setting up of the computer programmes involves considerable time and effort. The present method uses a simple U/B against dose transform which enables formulae to be derived which are relatively easy to program on a computer.

Suitable conditions of solid phase radioimmunoassay enable this simple transform to yield approximately linear dose response curves, and this has made it possible to devise a relatively simple method for the determination of E_2 in plasma. Although not programmed for optimal assay precision and sensitivity the derived formulae enable assay results to be obtained which are in good agreement with the normal solid radioimmunoassay system[4]. The method obviates the need for lengthy counting times for recovery assessment, and the need for full standard curves, thus saving assay time. Further assay time is saved by using specific antisera to E_2[5] circumventing the need for chromatographic separation.

EXPERIMENTAL

Symbols

x = dose (non radioactive), pg
E = dose (radioactive) pg
S = dose (non radioactive standard), pg
y^1 = recovery via extraction prior to assay. 100% recovery = $y^1 = 1{\cdot}0$
U = unbound d.p.m.
B = bound d.p.m.
m = slope of U/B against total dose ($x+E$)
U_B = unbound d.p.m. of blank (assay buffer)
B_B = bound d.p.m. of blank (assay buffer)
U_S = unbound d.p.m. of blank + standard dose (S)
B_S = bound d.p.m. of blank + standard dose (S)
U_P = unbound d.p.m. of plasma + blank
B_P = bound d.p.m. of plasma + blank
$R_B = U_B/B_B\,R_S = U_S/B_S\,R_P = U_P/B_P$

$$R_{BE} = \frac{U_B}{B_B(U_B+B_B)} \quad R_{SE} = \frac{U_S}{B_S(U_S+B_S)} \quad R_{PE} = \frac{U_P}{B_P(U_P+B_P)}$$

V = volume of plasma used for extraction, ml
x_B = blank E_2, pg
x_P = plasma E_2, pg
C = constant = intercept of U/B against dose for zero dose
n = number of binding sites per molecule of antibody = 2
P = concentration of antibody (moles/l)
K = intrinsic association constant of antibody hapten-binding (l/mole)

DERIVATION OF FORMULAE

1. *Prediction of assay result from dose response curve (no extraction)*

The usual steroid radioimmunoassay standard curve plot is % B against mass, i.e. % $B/(U+B)$ against dose. Such plots are non linear. The reciprocal $(U+B)/B$ plot over the same dose range can with suitable dilution of antisera and controlled conditions of assay (see below) give a plot which approximates to a linear dose

response. Representing this straight line by the usual equation $y = mx^1 + c$

$$(U+B)/B = m(x+E)+c.$$

The solid radioimmunoassay system appears to involve antibody hapten binding which behaves according to the law of mass action thus

$$\text{Lim} = 1 + 1/nPK$$
$$m(x+E) \to 0$$
$$\therefore c = 1 + 1/nPK$$

and

$$(U+B)/B = m(x+E) + 1 + 1/nPK,$$

i.e.

$$U/B = m(x+E) + 1/nPK. \tag{1}$$

In those cases of assay where $1/nPK$ is not small or negligible this would affect the blank x_B and a distinction would have to be made between the true blank involving this factor and the blank which ignored it. We have found in practice using the solid phase radioimmunoassay described in this communication that plots of U/B against the dose $(x+E)$ can be easily arranged to be approximately linear and they appear with very little approximation to pass through the origin (Fig. 1). The value of $1/nPK$ is therefore small using the solid phase radioimmunoassay system. This is probably due to the fact that not only is the value of K high [4×10^9 l/mole (Ref. [5])] but the concentration of binding sites nP is also high since it is a layer over a small area of polystyrene tube surface [7]. Thus we can ignore $1/nPK$

$$\therefore U/B = m(x+E) \qquad \text{(Fig. 1).} \tag{2}$$

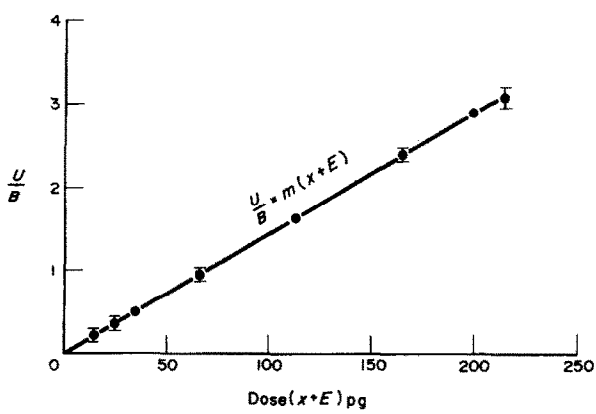

Fig. 1. Typical U/B against dose curve.

Simplifying for x

$$x = (1/m)\ (U/B) - E. \tag{3}$$

Thus if m is known, dose values can be calculated without reference to dose response curves, since E and the metameter U/B are known. Addition of a known standard S to the blank enables m to be calculated since $m = (R_S - R_B)/S$.

Substituting for m in equation (3) gives

$$x_B = S\left[\frac{R_B}{R_S - R_B}\right] - E \quad x_P = \frac{S}{V}\left[\frac{R_P - R_B}{R_S - R_B}\right]$$

Since using direct radioimmunoassay (no extraction) $U_B = (E - B_B)$ and $U_S = (E - B_S)$ then the above expressions can be determined in terms of E (known addition) and bound d.p.m. (B) only. Thus the popular double antibody precipitation method used for pituitary hormone radioimmunoassay can be used without reference to standard curves since

$$x_B = S\left[\frac{B_S(E - B_B)}{E(B_B - B_S)}\right] - E \tag{4}$$

$$x_P = \frac{S}{V}\left[\frac{B_S(B_B - B_P)}{B_P(B_B - B_S)}\right]. \tag{5}$$

Similar expressions using $B_B = (E - U_B)$ and $B_S = (E - U_S)$ enable the results to be expressed in terms of E and unbound counts only.

2. *Radioimmunoassay with extraction*

Equation (2) above (Fig. 1) has to be qualified for steroid radioimmunoassay and other methods involving extraction because the recovery y^1 is involved. In this case

$$U/B = my^1(x + E). \tag{6}$$

Since $y^1 = (U + B)/E$ (expressed as d.p.m. or mass) simplifying for x gives

$$x = \left[\frac{E}{m}\frac{U}{B(U + B)}\right] - E. \tag{7}$$

As previously, addition of a known standard S to the blank enables m to be found because

$$m = E/S\ (R_{SE} - R_{BE}).$$

Substituting for m in equation (7)

$$x_B = S\left[\frac{R_{BE}}{R_{SE} - R_{BE}}\right] - E \quad x_P = \frac{S}{V}\left[\frac{R_{PE} - R_{BE}}{R_{SE} - R_{BE}}\right].$$

Thus written in full for computer calculation of these formulae (formulae method)

$$\text{Blank } x_B(\text{pg}) = S\left[\frac{\dfrac{U_B}{B_B(U_B+B_B)}}{\dfrac{U_S}{B_S(U_S+B_S)}-\dfrac{U_B}{B_B(U_B+B_B)}}\right]-E. \tag{8}$$

$$\text{Plasma } x_P(\text{pg/ml}) = \frac{S}{V}\left[\frac{\dfrac{U_P}{B_P(U_P+B_P)}-\dfrac{U_B}{B_B(U_B+B_B)}}{\dfrac{U_S}{B_S(U_S+B_S)}-\dfrac{U_B}{B_B(U_B+B_B)}}\right]. \tag{9}$$

Linearity of dose-response curve

Any use of the above formulae for daily assays has to be based on the main assumption that the dose-response curves will remain approximately linear from day to day. Based on two points only (includes zero point) any theory of linear regression cannot account from curvature between these points. Plotting full standard curves (7 duplicates over the required 0–200 pg range) over a period of 2 weeks using the same batch of coated polystyrene tubes showed that although there was a range (±25%) in the daily magnitudes of m, all the dose-response curves were approximately linear.

The magnitude of m and linearity depend on those conditions affecting the U/B ratio. Using the controlled temperature and equilibration conditions described in this paper (see methods), it was found that the main variable affecting the linearity of response was that of dilution of antisera, the magnitude of the radioactive dose E having only a relatively small effect provided this dose was < 20 pg. The simultaneous coating of a batch of 250 tubes with the same dilution of antisera, (see methods) ensures constancy of antisera dilution for each batch. The tubes if stored at 4° at pH 6·9–7·1 and covered with parafilm to prevent evaporation, show little deterioration if any for a period up to a month[4]. Fortunately again the dilution of antisera was not highly critical, gross changes seriously affected the linearity of the dose-response curve whilst small changes had little effect.

The best way to find the conditions for a linear dose-response relationship is to use antisera at various dilutions using a fixed < 20 pg radioactive E dose. Using various batches of E_2 antisera (specific and non specific) at various dilutions it was found that the better approximations to a linear dose relationship were obtained when the U_B/B_B ratio for the blank possessed values between 0·25 and 0·66, and when the U_S/B_S ratio for the 200 pg standard dose point had values between 2·5 and 4·0. The linearity of most plots did not hold for doses > 200–225 pg, the value of m becoming progressively smaller. Again with low U/B ratios < 0·20 plots tended to curve. Sensitivity of measurement was achieved by using U/B ratios of 3·0–4·0 for the 200 pg standard dose.

Experiments showed that there was little difference in dose response curve linearity and in the magnitude of m, when either a 7·5 or a 15 pg radioactive dose was used. These experiments also show that there was little difference in m when the variable radioactive doses (y^1E) obtained after extraction are used for the various U/B metameters when the recovery y^1 varies between 0·4–0·6 for blanks + standards and 0·3–0·5 for plasma (see results). In practice it was found that the linearity of the dose response curve remained reasonably constant for any partic-

ular batch of coated polystyrene tubes (see methods). A check for the desired linearity using full standard curve doses was made with every new batch of tubes coated. A dilution of 1/20,000 $LR_4(6KE_2)B_2$ antisera[5] and a radioactive dose (E) of 15 pg satisfied the requirements for the linear dose response relationship by which the results reported in this paper were obtained.

MATERIALS

Water: all water was deionised and redistilled from glass.

Ethyl alcohol: this was refluxed over calcium hydride (1 h) and redistilled.

Dioxane: dioxane technical (BDH chemicals, Poole, Dorset, UK) was distilled after first refluxing over sodium (2 g per 5 l) for 1 h.

Ether: (BDH chemicals, Poole Dorset, UK) was redistilled before use.

Barbital buffer: this buffer (0·07 M sodium barbitone pH 9·8) was used as the medium for coating antibodies to polystyrene tubes.

Polystyrene tubes: small disposable 0·6 ml volume polystyrene tubes (LP/2, Luckham Ltd., Surrey, UK) were used for solid radioimmunoassay. Tubes were cleaned in a Decon solution (2% v/v), rinsed out with distilled water and dried in an oven at 50° before use.

Assay buffer: the assay buffer was similar to that previously described by Abraham[4]. Two stock solutions of sodium phosphate buffer were prepared. Solution A was 27·6 g $NaH_2PO_4H_2O$ per l and solution B was 53·65 $Na_2HPO_4 \cdot 7H_2O$ per 1. The assay buffer was prepared by adding 195 ml of solution A, 305 ml of solution B, 1 g of sodium azide, 9 g of sodium chloride to 500 ml water. The unadjusted pH was usually 7·0 (range 6·9–7·1). Good water was necessary to achieve this unadjusted pH.

Oestradiol-17β (E_2): this steroid was obtained from Koch-Light, Colnbrook, Bucks, UK.

Antisera: antisera to E_2 (BSA absorbed and Rivanol treated) was prepared in our laboratory as previously described[6]. The actual batch used was LR_4-$(6KE_2)B_2$. (See Ref. 5).

Radioactive oestradiol-17β (S.A. 100 Ci/m mol) (2, 4, 6, 7^{3}H.) from the Radiochemical Centre, Amersham, UK was checked for radiochemical purity by thin layer chromatography. Circa 0·5 mCi quantities were dissolved in ethyl alcohol (100 ml) as strong stock solution. The stock radioactive solution was used to prepare:

(i) A radioactive recovery solution. This solution contained 1760 d.p.m. (mass 2·2 pg)/0·1 ml assay buffer equivalent to 800 c.p.m. in toluene scintillator, which was used for recovery assessment in the normal solid radioimmunoassay technique.

(ii) A radioactive dose solution (E). This solution contained 12,000 d.p.m. (mass 15·0 pg)/0·1 ml assay buffer (circa 5400 c.p.m. in toluene scintillator).

(iii) A standard solution (S). The solution consisted of the radioactive dose (ii) above plus 100 pg non-radioactive E_2 in 0·1 ml assay buffer.

Toluene Scintillator. This scintillator was made up by adding 2·5-diphenyloxazole (PPO) (3·0 g) to 1·4-bis 2-(4-methyl-5-phenyloxazolyl benzene (dimethyl-POPOP) (0·3 g) in reagent grade toluene (1 l).

Dioxane scintillator was made up by adding PPO (7 g), naphthalene (100 g) and dimethyl-POPOP (0·3 g) to dioxane (1 l). A Tri-Carb Liquid Scintillation Spectrometer Model 3375 (Packard Instruments Ltd.) was used for all radioactive

measurements; counting efficiency was 45% for tritium in the toluene scintillator and 35% in the dioxane scintillator. Radioactivity measurements were made to within 2% accuracy.

METHODS

Preparation of antibody coated tubes. The small polystyrene tubes were placed in a vertical position in a rack designed to hold 250 tubes. Antisera LR_4-$(6KE_2)B_2$ was diluted to a final dilution of 1/20,000 with barbital buffer (for conditions of dilution for any antisera see above). 0·5 ml of the diluted antisera was added to the polystyrene tubes, and the tubes incubated at 4° for 16 h overnight. After incubation and careful removal of the barbital buffer by suction, 0·5 ml of assay buffer was added which was again removed by suction. A further 0·5 ml of assay buffer was then added and the tubes covered with parafilm and stored at 4°C until use. Coated tubes were stable for at least a month kept at this temperature.

Radioactive dose. To meet the requirements for mass of radioactive dose (see above), it was decided to use a mass of 15 pg. The efficiency of counting of tritium using the toluene based scintillator was 45%, thus using E_2 with a specific activity of 100 Ci/m mol., a mass of 15 pg was 5400 c.p.m. or 12,000 d.p.m. A check on the linearity of the dose-response curve is made with each batch of tubes coated.

Solid phase radioimmunoassay technique with recovery count and standard curves

The formulae method using the computer was checked using plasma samples processed and determined by a solid phase radioimmunoassay technique using normal recovery count and standard curves. The method used was essentially similar to that of Abraham and Odell [7] with the following modifications to obtain reproducibility. The small LP/2 polystyrene tubes were used instead of the larger tubes used by these workers (see precision) and no chromatographic separation was used because the antisera was specific for E_2.

Antisera $LR_4(6KE_2)B_2$ was used at 1/20,000 dilution.

(A) *Extraction.* All samples were assayed in triplicate. Volumes of plasma and blank used were 0·5–2·0 and 0·1 ml of the radioactive recovery solution was added to all samples. When samples were less than 2 ml they were brought up to this volume with water and then all were extracted with 4 ml ether. The ether was then transferred by Pasteur pipette to a small pointed tube (Fig. 2) and blown to dryness under nitrogen. The residue was dissolved in 1 ml assay buffer. 0·2 ml was used for recovery assessment, and 0·4 ml transferred to precoated polystyrene tubes for radioimmunoassay.

(B) *Radioimmunoassay.* 6 Standards (in duplicate) to cover the range up to 200 pg were set up to obtain a standard curve. 0·1 ml of solution E (the radioactive dose) was added to all samples of blank plasma and standards, the tubes were gently shaken for 1 min and then left to incubate overnight at 4°. (16 h). After incubation the liquid in the tubes (unbound fraction = U) was transferred by a Pasteur pipette to a scintillation phial, and a further 0·5 ml assay buffer was added to the polystyrene tubes which was then also transferred to the phial. Radioactivity was counted using 10 ml dioxane scintillation fluid. Results were calculated in the normal way by assessment from the standard curve plotted as %-bound

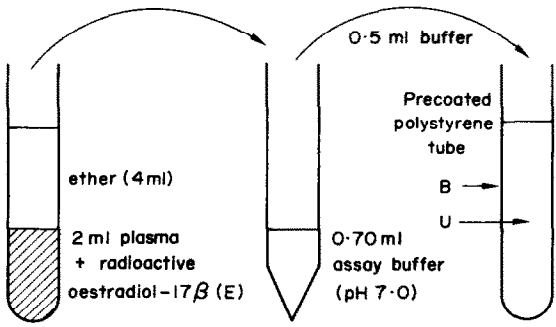

Fig. 2. Plasma (up to 2 ml) + radioactive dose E was extracted with ether, transferred to the pointed tube and blown to dryness under nitrogen. 0·70 ml assay buffer was then added and 0·5 ml of the dissolved residue transferred to the precoated polystyrene tube for radioimmunoassay.

against dose of standard used, and the usual correction made after counting samples for recovery.

The formulae method

The solid phase radioimmunoassay technique used for the method using the formulae (formulae method) in which full standard curves and recovery counts were discarded, and by which the formulae was processed by computer was as follows:

As with the previous method LP/2 tubes were used to obtain reproducibility and since $LR_4(6KE_2)B_2$ antisera was specific, no chromatography was involved. A 1/20,000 dilution for coating this antisera, when used under the conditions below, gave linear U/B against dose curves. (Fig. 1).

(a) *Extraction.* All samples were assayed in triplicate. Volumes (0·5–2·0 ml) of plasma and blanks were used plasma samples less than 2 ml were made up with assay buffer to this volume. Samples consisted of a blank, a blank + 100 pg E_2 standard, and numerous plasma samples. 0·1 ml of radioactive load solution E was added to all samples. 0·1 ml of standard solution S was added to one of the blank samples only (in triplicate). All samples were extracted with 4 ml ether. The ether was transferred by Pasteur pipette to a small pointed tube (Fig. 1) and then blown to dryness under nitrogen. The residue was dissolved in 0·70 ml assay buffer.

(b) *Radioimmunoassay.* An 0·5 ml aliquot of the assay buffer was transferred to a precoated polystyrene tube and incubated at 4°C overnight (16 h). After incubation the liquid in the tube (unbound fraction = U) followed by a further 0·5 ml assay buffer wash was quantitatively transferred to a scintillation phial containing 10 ml dioxane scintillation fluid and the radioactivity counted. The bound fraction B (wall of polystyrene tube) was also counted. The tube was dropped into 10 ml toluene scintillator in which it completely dissolved within 30 min. The c.p.m. results were then processed by a computer programmed to calculate the formulae for blank and respective plasma samples using the formulae for x_B and X_P (equations 8 and 9).

Calculation of results by computer

Results were obtained by calculating equations (8) for the blank and equation

(9) for the respective plasma samples (see derivation of formulae). Calculations were made using a 6CDC (London University) computer (programmed in Fortran), but they could easily be made on a simple calculating machine. The respective B and U c.p.m. values together with their respective external standard ratios (AES) obtained from the teleprinter attached to the Tri-Carb Scintillation Counter were processed on the computer to give d.p.m. values. This conversion was necessary because the quenching of the aqueous fraction (U) in dioxane based scintillator was greater than that of the tubes (B) assessed in toluene, with the result that incorrect R values are obtained when B and U are assessed as c.p.m. Since the graphs of AES against % efficiency used for correction of quenching were linear for both scintillators over the range of measurement this enabled the d.p.m. to be readily calculated since d.p.m. $= 100$ c.p.m.$/m$(AES $- C$) where $m =$ slope of AES/% efficiency graph for the respective scintillator, and $C =$ respective intercept.

The B and U d.p.m. data was then programmed so that the computer could calculate the blank x_B by equation (8). Data for S and E was fed directly into the computer as pg mass and V as ml. After calculation of the blank the denominator and its constant ($R_{SE} - R_{BE}$) which is common to both equations was stored for subsequent calculation of all plasma results involving their respective U_P and B_P d.p.m. values.

The computer was programmed with safeguards to ensure that erroneous data was not processed. The R_B values were small in comparison with R_S and R_P values, so the computer was programmed to allow any R_B results with a standard deviation (S.D.) up to $\pm 30\%$ from the mean of this triplicate group before being discarded. The R_S and R_P values however were allowed $\pm 10\%$ from the mean before these were discarded. The computer was first presented with 3 results. It obtained the mean of these results and a standard deviation (S.D.). If the S.D. did not fall within the above prescribed limits, the result having a value farthest from the mean was discarded, otherwise if it satisfied the above criteria all three results were accepted. When only 2 results remained, the computer calculated their mean and S.D., and if this S.D. fell within the required limits accepted them. If not they were processed as not acceptable, the computer being programmed to provide a warning to this effect. Provided the R_S and R_B values fell within the prescribed limits the assay could proceed and only individual plasma group results not having the required reproducibility were discarded. Duplicate plasma samples were sometimes used as well as triplicates reported in the results. The computer was programmed to inform of the number of determinations in the group used for calculation of each respective R value and to print out the respective S.D. Most groups gave results which had S.D. well below $\pm 10\%$ required for acceptance. The computer was not only programmed to give an S.D. for each triplicate group, but also to reveal information as to whether this S.D. was calculated for dose $E > 200$ pg. All results calculated with a total dose more than 200 pg had their dose value printed out. This limited dose (minus circa 7·5 pg E, and 5 pg blank dose) represented a plasma value up to about 750 pg/ml, to be calculable on the linear portion of the curve, when 0·5 ml plasma was assayed with 50% recovery.

RESULTS

Precision. Good precision between individual groups of triplicate and duplicate determinations was obtained using both solid phase radioimmunoassay

techniques. The average coefficient of variation for standard curve duplicates was ±3·7% ($n = 68$) for a range of 10–200 pg oestradiol-17β standards. The average coefficient of variation for plasma triplicates was ±4·9% ($n = 18$) for a range circa 50–150 pg/ml, and was ± 10·8% ($n = 18$), range circa 5–20 pg for blanks. (see columns A and B, Table 1).

Standard curves. Fig. 1 shows a typical standard curve using antisera LR_4-$(6KE_2)B_2$ at 1/20,000 dilution for coating the LP/2 polystyrene tubes. The plot is U/B against total dose of oestradiol-17β (including the radioactive dose E). The figure shows that the plot approximates a straight line up to about 200 pg, justifying the use of the formulae up to this dose.

Table 1. Comparison of results obtained by use of standard curves and recovery assessment (A) with the formulae using a computer (B).

(A) Standard curve	Blank (pg) (B) Formulae	(C) Mean of methods
5·1 ± 0·3 (5·9)	11·3 ± 0·5 (4·4)	8·2 ± 3·0 (36·5)
7·1 ± 1·0 (14·1)	7·3 ± 1·5 (20·5)	7·2 ± 0·1 (1·4)
11·7 ± 0·2 (1·7)	13·4 ± 1·0 (7·5)	12·5 ± 0·8 (6·4)
12·1 ± 1·3 (10·7)	9·1 ± 1·0 (10·9)	10·6 ± 1·5 (14·2)
6·5 ± 0·1 (1·5)	8·3 ± 0·2 (2·4)	7·4 ± 0·9 (12·2)
8·4 ± 0·9 (10·7)	9·6 ± 0·9 (9·3)	9·0 ± 0·6 (6·6)
14·4 ± 4·0 (27·7)	10·5 ± 3·0 (28·5)	12·5 ± 2·0 (16·0)
20·6 ± 3·4 (15·5)	16·9 ± 2·9 (17·2)	18·6 ± 1·7 (9·1)
4·6 ± 0·1 (2·2)	2·8 ± 0·1 (3·5)	3·7 ± 0·9 (24·3)
Mean = of group		
10·0 ± 1·0 (10·1)	9·9 ± 1·1 (11·6)	10·0 ± 1·4 (14·1)
	Plasma (pg/ml)	
63·1 ± 5·3 (8·4)	62·8 ± 5·5 (8·7)	62·9 ± 0·1 (0·2)
60·1 ± 1·5 (2·5)	60·6 ± 2·3 (3·8)	60·4 ± 0·3 (0·5)
49·7 ± 0·3 (0·6)	51·6 ± 0·2 (0·4)	50·7 ± 1·0 (2·0)
93·2 ± 2·0 (2·1)	82·5 ± 2·0 (2·4)	88·0 ± 5·3 (6·0)
99·4 ± 9·0 (9·1)	96·0 ± 7·6 (7·9)	97·8 ± 1·7 (1·7)
45·1 ± 5·4 (12·0)	45·9 ± 4·3 (9·4)	45·5 ± 0·4 (0·9)
142·5 ± 4·4 (3·1)	125·9 ± 3·2 (2·5)	134·2 ± 8·4 (6·2)
131·0 ± 4·2 (3·2)	116·9 ± 4·8 (4·1)	123·9 ± 7·0 (5·7)
113·9 ± 4·7 (4·1)	106·5 ± 4·1 (3·8)	110·2 ± 4·1 (3·7)
Mean = of group		
88·4 ± 4·4 (5·0)	83·1 ± 4·0 (4·8)	85·9 ± 2·6 (3·0)

Each result is a mean ± S.D. of triplicate determinations.
Figures in brackets are coefficients of variation.

Predictability of the standard curve by the formulae method. 15 groups of triplicates of the blank (2 ml assay buffer) and 15 groups of triplicates to which 100 pg oestradiol-17β was added were determined by the method using the formulae. A computer was used specially programmed to calculate the m values (m = slope of line of U/B against dose = $(E/S)(R_S - R_B)$. A standard curve (plotted as U/B against total dose) was determined as normally without extraction

alongside each individual group set (blanks and blanks + 100 pg standard). Each standard curve and the two respective groups were determined simultaneously. Standard curves covered the range 10–200 pg, duplicate estimations were made of each standard dose. A comparison was made of the m values calculated by the two methods. Despite the day to day variation of m, excellent agreement between the two methods was obtained with the simultaneous assays, the average difference between the mean values of the methods was $\pm 3{\cdot}9\%$ ($n = 15$). These results indicate that slope of the standard curve (U/B against dose) could be determined in spite of the variable recoveries (40–60%) via the extraction method. A similar degree of agreement ($\pm 5\%$) was obtained from a few experiments in which the 100 pg standard was added to plasma instead of the blank. This experiment indicated that the value of m did not change to any appreciable extent despite the presence of plasma constituents during the extraction, justifying the use of the normal standard curve in radioimmunoassays (standards not added to plasma or even put through the extraction procedure).

Comparison of blank and plasma results obtained by the normal standard curve and recovery method with the formulae method using a computer. A comparison of results obtained by the standard curve method (A) and the formulae method (B) for identical blank and plasma samples is given in Table 1. Results are in good agreement. The table shows (column C) that the mean difference between the two methods for 9 groups of triplicate blank determinations is 14·1% with an average blank of 10 pg ($n = 54$) range (4·6–20·6 pg) and the mean difference between the methods for 9 groups of triplicate plasma determinations is $\pm 3{\cdot}0\%$ for the range 45·1–142·5 pg/ml.

The average recovery via the standard curve method (the actual amount assayed) was 30–35% for a blank and 20–25% for plasma, whilst the mean recovery via the formulae method was 50% (range 40–60%) for blanks and 40% (range 30–50%) for plasma.

DISCUSSION

The results (Table 1) show that reasonably good precision can be obtained by the solid phase radioimmunoassay technique. This technique has unfortunately not been as widely used as the liquid radioimmunoassay approach[8] due to its previous poor reproducibility. In certain cases the solid phase radioimmunoassay has been abandoned in favour of the liquid phase technique[9]. A previous comparison conducted in our laboratory between polystyrene tubes (Falcon Plastics, Los Angeles, Calif.) used by Abraham and Odell[7] with the LP/2 tubes from England, suggested that the latter tubes were far more reproducible as regarding consistency of area of coating of antisera. Again the extra 0·5 ml of assay buffer used to wash out the unbound fraction U after equilibration of the radioimmunoassay also contributed towards greater reproducibility. Of all the methods of radioimmunoassay available, none appears as simple as the solid phase radioimmunoassay technique since none offers the unique feature of accomplishing equilibration and separation of free from bound steroid in a simple single step.

Assessed on d.p.m. or mass, the value $(U + B)$ in the present method represents the recovery after extraction, since if the radioactive dose E is known in similar terms then recovery $y^1 = (U + B)/E$. Knowledge of $(U + B)$ is readily available using the formulae solid phase radioimmunoassay method, and since E has a known value, the method obviates the need for the usual recovery count.

This approach has a distinct advantage over existing steroid radioimmunoassay procedures, in that it obviates the need to add the usual small number of c.p.m. prior to extraction, and then take aliquots for recovery assessment prior to the radioimmunoassay proper. The radioactive counting of the recovery aliquots in existing procedures is probably the most time-consuming step, since to count relatively low c.p.m. (often 100 c.p.m. or less) with accuracy necessitates very long counting times. In contradistinction to other techniques, the present method enables the recovery to be assessed after the actual radioimmunoassay step, thus eliminating errors due to transferring aliquots prior to this stage and multiplication of aliquot errors in the recovery assessment. Despite the fact the present technique counts both the B and U fractions instead of just the U fraction, it only involves the same number of samples for counting as other methods, since no recovery count is necessary. The great advantage of the present method is that a much larger c.p.m. is available for counting in any fraction, leading to much shorter counting times to achieve the required accuracy of c.p.m. assessment. The method not only dispenses with the recovery aliquot count, but also enables a much quicker assessment of the final result.

The present method dispenses with the use of full standard curves. Instead a 100 pg standard is added to 3 blanks, and this enables m to be calculated, and to define the line representing the standard curve. Only 3 extra samples are required by the method compared with the usual set of duplicate standards for 6 or 7 doses (12–14 samples) used for a typical standard. This saves assay time. Adding the 100 pg standard to plasma enabled similar values of m to be determined as when it was added to the blank, showing that m is not changed to any appreciable extent by the presence of plasma constituents during the extraction, justifying the addition of the standard to the blank, and the use of standard curves without extraction and addition of the various doses to plasma. Since the plasma E_2 levels can vary considerably (especially across the menstrual cycle) the standard is added to the blank to ensure that the 200 pg dose limit is not readily exceeded, because at this dose the dose-response curves commenced to be non-linear.

Any immunological method is only as specific as the antisera used in the determination. The present method has used the antisera reasonably specific for E_2 of Exley *et al.* [5]. Proof that the antibody is reasonably specific (its only major cross reaction $> 2\%$ is with C_6 substituted E_2) is borne out not only by cross specificity tests by these authors and other workers, but by comparing results obtained for normal female plasma with and without thin layer chromatographic separation [10] when the regression line for samples involving thin layer chromatographic separation (y) and direct extraction without chromatographic separation (x) was $x = 0{\cdot}94y + 5{\cdot}8$ ($n = 43$). The present method could be performed using less specific antisera, but like all other methods this would necessitate chromatographic separation. The ease of operation of the methodology given in this communication is partly determined by the fact that it involves no chromatographic separation, due to the quality of antisera used.

The use of the formulae and computerisation is applicable to any radioimmunoassay procedure provided correct B and U values can be measured after equilibration and provided the dose-response curve can be approximated to be linear, and the curve passes through the origin. Once it was ascertained that the technique of solid radioimmunoassay could be made to give reasonable precision, this was the method of choice because of its ease of operation compared with

other methods. Experiments conducted in this laboratory have indicated that free E_2 (U fraction) adsorbed on dextran-coated charcoal in liquid phase radioimmunoassay[11] can be readily extracted by water followed by dioxane scintillator, thus enabling both B and U c.p.m. values to be obtained with this system. Other experiments have indicated that an approximate linear dose-response can be obtained using E_2 antisera and the liquid phase radioimmunoassay, suggesting that this method may be amenable to formulae data computerisation. The approach may also be applicable to direct radioimmunoassay methods (i.e. no extraction prior to assay). In this case the simplified formulae derived in terms of E and U, or E and B could be used. The popular double antibody precipitation technique used in pituitary hormone radioimmunoassay which counts B only may be a good candidate for this approach. Using the condition of linear dose-response the approach may be possible with any steroid or any antigenic material (haptenic or non-haptenic), and with many radioligand assays.

The chief advantage of the present method is its ease and rapidity of operation. Discounting the overnight equilibration which was chosen for convenience the actual working time on the bench is very short once a large batch of polystyrene tubes coated with antisera have been prepared. The 3 blanks, 3 blanks + standard together with the plasma samples in duplicate or triplicate can be quickly extracted, and the 0·5 ml assay buffer containing the residues is readily applied to the coated tubes. Next morning separation of the B and U fractions is quickly achieved. After 5 min counting of radioactivity, the results from the Tri-Carb Scintillation Counter are fed directly via the attached teleprinter on tape to the computer programmed to process the formulae. In the event a computer is not available the calculation can easily be made on a simple calculating machine. If there is a requirement to obtain the results on the same day, the 2–4 h equilibration technique[7] could be considered.

A preliminary report of this work published in abstract form[14] was presented at the IV International Congress of Endocrinology held at Washington DC, USA, 1972.

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