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FURTHER STUDIES ON RADIOIMMUNOASSAY SYSTEMS FOR PLASMA OESTRADIOL

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SUMMARY

A systematic study has been made of alternative procedures in a liquid phase radioimmunoassay system for the determination of oestradiol in peripheral venous plasma from men and women. Accordingly, the relative merits of two radionuclides, three antisera, four separation techniques and three methods of measuring radioactivity have been determined.

The results show that the most accurate values are obtained if an antiserum to oestradiol-6-carboxymethyl oxime is used, and that a chromatographic step is not required for routine assays. In addition, the values for precision, sensitivity and accuracy are similar, whether [2,4,6,7-^{3}H]-oestradiol or oestradiol-6-carboxymethyl oxime-mono^{125} iodohistamine is used as labelled tracer. Marginally lower (<10%) values may be obtained if a disequilibrium technique is used with tritiated oestradiol. The lowest values with either labelled derivative are found if a mixture of ammonium and calcium sulphates is used to separate the antibody-bound fraction, but this finding is associated with the highest degree of non-specific binding (approx. 30%). However the level of non-specific binding with this, and the other techniques studied, is independent of the mass of oestradiol in the system (0–200 pg) and the temperature at which the assay is performed (4 or 37°C). If a separation technique is chosen such that β counting may be performed in the assay tube, then the cost effectiveness regarding the use of tritium or iodine-125 depends upon the number of assays performed in a given time. Thus under the conditions described, the total costs would be similar over a period of 1 yr in which more than 3000 samples were processed.

INTRODUCTION

Since 1969 many methods based upon the principles of radioimmunoassay have been applied to the determination of oestradiol [1,3,5,(10)-oestratriene-3,17β diol] in peripheral venous plasma from men and women [1–18]. During this period numerous innovations and refinements have been introduced into the basic procedure, notably with respect to overall specificity [18–24] and a reduction in assay time.

The present communication is concerned with an in depth analysis of the various steps in a liquid phase assay system, and an examination of factors that affect the choice of alternative techniques or conditions. The results from these studies have led to the development of practical procedures for the optimization of standard curves, and the selection of optimum conditions for the overall sensitivity and accuracy of the method.

EXPERIMENTAL

Solvents and reagents

Acetone, benzene, ethanol, diethyl ether, methanol and water were all Analar grade and used without further purification. Sephadex LH 20 and Dextran T70 were obtained from Pharmacia, Uppsala, Sweden. Isatin (2,3-indolinedione) and Triton X-100 were purchased from Koch–Light Laboratories Ltd, Colnbrook, Bucks, England.

Buffer

Tricine [*N*-Tris (hydroxymethyl) methylglycine] buffered saline (TBS) containing 0·1% gelatine was prepared by dissolving 9 g sodium chloride, 1 g sodium azide and 1 g of gelatine in 1 litre of 0·1 mol/l Tricine in Analar water. The pH was adjusted to 8·0 with 10 N sodium hydroxide and the solution stored at 4°C.

Standards

Samples of oestrone [1,3,5(10)-oestratrien-3-ol-17-one], oestradiol and oestriol [1,3,5(10)-oestratriene-3, 16α, 17β-triol] were obtained from Sigma Chemical Co, St. Louis, Mo, U.S.A., and recrystallized in appropriate solvents before use. Small amounts (approx. 10 mg) of authentic oestradiol-17α, oestradiol-17β-glucuronoside, oestradiol-17β-sulphate, oestradiol-3-glucuronoside and oestradiol-3-sulphate were donated from the MRC Steroid Reference Collection, Westfield College, London, N.W.3. Samples of 6-oxo-oestradiol [1,3,5(10)-oestratrien-3, 17β-diol-6-one] and oestradiol-3-hemisuccinate were purchased from Steraloids INC. Pawling, N.Y. 12564, U.S.A.

[2,4,6,7-^{3}H]-oestradiol, S.A. 100 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, Bucks, England. A solution was prepared in benzene–ethanol (95–5 v/v) such that each ml contained 20 μCi. Before use 50 μl are removed, thoroughly dried and redissolved in 11 ml of TBS. One hundred microlitres of this solution, containing approx. 20,000 d.p.m. (25 pg of steroid) is used in the assay system. An internal standard was prepared by diluting 5 μl of the original solution in 10 ml of water so that 100 μl contain approximately 2000 d.p.m. (2·5 pg).

Oestradiol-6-carboxymethyl oxime was prepared from 6-oxo-oestradiol according to the method of

Erlanger *et al.*[25]. Histamine was labelled with iodine-125 and linked to oestradiol-6-carboxymethyl-oxime by the method described by Hunter *et al.*[26]. The products were purified by t.l.c. on silica gel in the system benzene–ethanol, 75:25 v/v. Oestradiol-6-carboxymethyl oxime-mono-iodohistamine was located by autoradiography, and eluted with acetone. The eluate was diluted such that 100 μl contained 2,000,000 c.p.m. (400 pg). Prior to use 100 μl of this solution is taken, dried, and redissolved in 10 ml of buffer. One hundred microlitres (20,000 c.p.m., 4 pg) is used in the assay system.

Antigens

Batches of oestradiol-3-hemisuccinate, oestradiol-6-carboxymethyl oxime and oestradiol-17β-hemisuccinate were linked to BSA according to the procedure described by Erlanger *et al.*[25].

Immunization procedure

The respective antisera were raised in rabbits, pretreated with an intradermal injection of 750 μg of dried tuberculin bacilli (Difco Laboratories, West Molesey, Surrey, England). After 4 weeks, 100 μg of antigen per animal were dissolved in 400 μl of saline, emulsified with 800 μl of Freund's complete adjuvant and injected subcutaneously at multiple sites. This procedure was repeated once a week for a further 3 weeks, and the animals were bled from a marginal ear vein 10 days after the last injection. Subsequently, booster doses of antigen were given at monthly intervals, or when a fresh sample of serum was required. Using this approach, and at least four animals per antigen, reasonably specific antibodies with a high titre and avidity were obtained within 6 months.

Antisera

The samples of antisera were diluted 1:1 with a solution of 0·2% sodium azide, and stored at 4°C. In addition, batches of antisera to oestradiol-11α-succinyl-BSA (donated by Dr. A. H. Schuurs, Biochemical R & D Labs., N.V. Organon, Oss, Holland) and oestradiol-6-carboxymethyl oxime-BSA (donated by Professor H. R. Lindner, Department of Biodynamics, The Weizmann Institute of Science, Rehovot, Israel) were treated in a similar manner. The final dilutions of antisera were made immediately before use.

Dextran–coated charcoal (DCC)

Norit A charcoal from Sigma Chemical Co, St. Louis, Mo., U.S.A., was washed with water until all the fine particles had been removed. The purified material was dried at 120°C. Dextran T70 was dissolved in water (containing 0·1% sodium azide (v/v) to a final concentration of 100 mg/ml). The Norit A charcoal (5 g) and Dextran T70 (5 ml) were added to 1 litre of TBS. The solution was stored at 4°C. During use the charcoal was maintained in suspension by a magnetic stirrer.

Ammonium sulphate; calcium sulphate (ACS)

A solution of ammonium sulphate (65% saturated) was prepared by dissolving 295 g of ammonium sulphate and 1 g of sodium azide in 750 ml of water. The pH was adjusted to 8·0 with 10 N sodium hydroxide and the solution stored at 4°C. Immediately prior to use 1 g of powdered calcium sulphate (dihydrate) is added for each 25 ml of ammonium sulphate solution. The solid is dispersed with ultrasound; and during sampling an even suspension is maintained by the use of a magnetic stirrer.

Polyethylene glycol (PEG)

Thirty grams of polyethylene glycol (Mol. wt. 4000) was dissolved in 100 ml of water containing 0·1% sodium azide, and the solution was stored at 4°C. Prior to used calcium sulphate was added in the same proportion and manner as with ACS.

Double antibody (DA)

An antiserum (RD17) raised in the donkey to rabbit globulin was purchased from Wellcome Reagents Ltd, Beckenham, England, BR3 3BS. The solution was added to the assay system at a final dilution in buffer of 1:120 (v/v) with non-immune rabbit serum diluted 1 in 1200 (v/v). This combination gave a good precipitation plateau when the reactants were incubated overnight at 4°C.

In some experiments the reagent was added after the primary reaction had reached equilibrium. However, the best results were obtained when the second antibody was added to the first antibody for at least 16 h at 4°C before addition of the authentic and labelled steroids (the pre-precipitation technique). Prior to use the double antibody complex was maintained in suspension with a magnetic stirrer.

Radioactivity measurement

The scintillation fluid (SF) was prepared by dissolving 15 g of 2,4-diphenyloxazole (PPO) in 2 l. of toluene, and subsequently adding 1 litre of Triton X-100. The absolute amount of radioactivity in every sample was determined either in a conventional counting vial using 10 ml of SF, or in the assay tube using 1 ml of SF after resuspension of the precipitate [27, 28]. In both procedures the number of dpm is calculated by a channels ratio method with 4000 counts accumulating in the channel with the lowest count rate. The amount of iodine-125 in the assay tube was determined in the conventional manner using an automatic γ-counter.

METHODS

General description

A known volume of sample is taken, and if necessary an internal standard of tritiated oestradiol is added to enable experimental losses incurred during purification to be calculated, and the final results corrected accordingly. A standard curve is prepared over a suitable mass range, and appropriate aliquots are removed

Table 1. Some characteristics of iodine-125 and tritium with reference to oestradiol

Parameter	Iodine-125	Tritium
Type of emmission	γ and X-rays	β-
Energy (MeV)	0·035 0·033	0·018
Half-life (yrs)	0·164	12·3
Approx. cost (£/mCi)	10 as NaI^{125}	15 As oestradiol
Typical S.A. of labelled steroid (Ci/mmol)	5000	100
Binding to antiserum	$\gg$ Oestradiol	< Oestradiol
Type of counter	Solid scintillation	Liquid scintillation
Counting efficiency (%)	40–60	25–40

for assay. The antiserum and radioactively labelled compounds are added in buffer solution.

The sequence in which the reagents are added and the times of incubation depend upon the sensitivity required, the characteristics of the antiserum, and general convenience. After a suitable period of incubation, at a constant temperature, the antibody-bound and free compounds are separated, and the radioactivity is determined in either the bound or free fractions, or both. The concentration of oestradiol in the sample is calculated from the reading on the standard curve, the aliquot taken for assay, the recovery and mass of internal standard and the initial volume of the sample.

Choice of isotope

Tritium or iodine-125 [26, 29] have been advocated for use in radioimmunoassay systems for oestradiol. For reasons of stability no more than four hydrogen atoms in the steroid molecule (at carbon atoms 2, 4, 6 and 7) have been substituted with tritium. One potential advantage to using iodine-125 is that one atom of this isotope provides about 25 times the detectable counting rate given by four atoms of tritium. However, the direct substitutions of iodine-125 into the steroid molecule invariably cause an overwhelming loss of affinity for the antibodies. This problem has been overcome by linking the iodine-125 to a carrier molecule in the same position as the linking unit in the antigen. To date, tyrosine methyl ester, tyramine and histamine have been used for this purpose. These compounds either contain a phenol or an imidazole group for iodination by the chloramine-T reaction, and an amino group for coupling to the carboxyl group of the hapten by a mixed anhydride reaction. Some of the characteristics of tritium and iodine-125, which influence the choice between these two isotopes are listed in Table 1. The most obvious advantage of iodine-125 is that a gamma-counter may be used, and as the function of this instrument is based upon the use of a permanent inorganic scintillant, the cost of radioactive measurements is minimal. In addition, the higher specific activities obtainable (7000 Ci/mmol) would at first seem to indicate that smaller samples, and less solvent might be required, because of the smaller mass of labelled material. However, the iodinated derivatives usually have a greater affinity for the antibodies than authentic oestradiol, and this phenomenon invariably negates the advantage of the higher specific activity. There are certain disadvantages to the use of iodine-125. As the half-life is only 57 days, the iodine containing derivatives must by synthesized, purified and characterized at regular intervals. One disadvantage to the use of tritium is the possible occurrence of isotope effects. In this connection Jeffcoate *et al.*[30] have reported that [2,4,6,7-^{3}H]-oestradiol has only 40% of the potency of authentic oestradiol to compete with oestradiol-6-carboxymethyl oxime-histamine-I^{125} for binding sites to antibodies raised against an oestradiol-6-albumin conjugate. In the present study a direct comparison has been made between the competition of tritiated oestradiol and oestradiol, for binding sites on antibodies to oestradiol-6-carboxymethyl oxime-BSA, by preparing standard curves using labelled material only, and in the conventional manner using labelled and non-labelled material. In the former procedure solutions were prepared that contained 8, 4, 3, 2, 1·5, 1·25, 1·0, 0·75 and 0·5, times 20,000 d.p.m./100 μl of buffer. Each dilution was equilibrated with diluted antiserum and the unbound steroid removed by the addition of Dextran–coated charcoal. A standard curve was prepared by plotting the fraction of radioactivity bound times 20,000, on the ordinate, against the mass of oestradiol added minus the mass in 20,000 d.p.m. on the abscissa. If the results were compared with the curve produced by the addition of progressive amounts of oestradiol to 20,000 d.p.m. of tritiated oestradiol, then approximately 20% more tritiated material was required to reduce the initial binding by 50%.

Choice of antisera

The specificity of the antisera produced depends primarily on the position of linkage, and a retrospective analysis of the results shows that antibodies tend to discriminate most efficiently between compounds with changes in structure at atoms remote from the position of linkage on the steroid to the carrier molecule. Another factor to be considered is the relative concentrations of the immediate precursors and metabolites of oestradiol in the sample to be assayed. In this context, those sites on the molecule of oestradiol, which may be subject to metabolic change, and the positions of linkage that have been made to bovine serum albumin are shown in Fig. 1. Of the 18 carbon atoms in the steroid at least 6 have been joined to the carrier molecule. If carbon atoms 10 and 13 are excluded, then 10 positions, remain to be studied. The relative potency of various steroids to compete with

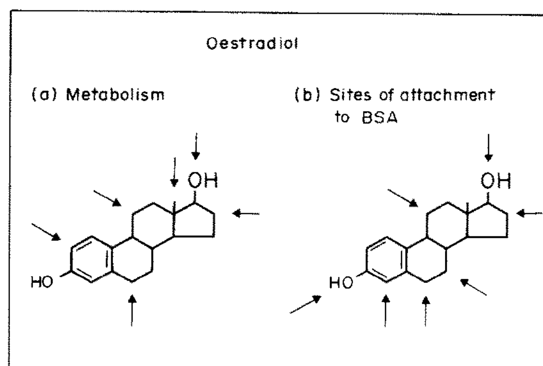

Fig. 1. The principal sites of metabolism for oestradiol, and the positions of attachment to a carrier molecule that have been used in the synthesis of antigens.

tritiated oestradiol for binding sites on the various antisera are listed in Table 2.

Extraction

Under ideal conditions the assay would be performed on untreated (or heat treated) plasma. In practice, however, these procedures result in a gross overestimate (>200%). Accordingly, various techniques have been devised and evaluated to extract oestradiol from material that might interfere with the assay. The most popular method involves the use of an organic solvent —usually diethyl ether. In this procedure, an internal standard of tritiated oestradiol (2000 d.p.m./2·5 pg) is added to 2 ml of every plasma sample, to 2 ml of water (method blank) and to two vials or assay tubes for liquid scintillation counting. The plasma samples and the blank are extracted twice with diethyl ether—once with 10 ml and then with 5 ml, using a vortex mixer. The ether layers are removed with a Pasteur pipette and the pooled extracts dried at 40°C under nitrogen.

Column chromatography

Although extraction is a purification procedure, it is often essential to purify the material further, or to check the result, by the inclusion of a chromatographic step prior to assay. One procedure that combines adequate resolution with reasonable blank values is chromatography on a column of Sephadex LH 20. Accordingly, the dried extract is dissolved in 100 μl of benzene–methanol 85:15 (v/v) containing 0·1% isatin. The extract is transferred to a column (10 × 0·5 cm.) of Sephadex LH 20 that has been equilibrated with benzene–methanol 85:15 (v/v). Elution is performed with the same solvent mixture and the yellow fraction containing the oestradiol is collected in a counting vial. The eluate is dried under nitrogen at 40°C and redissolved in 500 μl of buffer. Twenty per cent of the extract is removed in duplicate for assay, and a further 40% is subjected to liquid scintillation counting in order to determine the recovery.

Optimization of standard curve

A practical procedure for producing a highly sensitive standard curve as rapidly as possible is as follows:

1. Obtain a large volume of high titre, high avidity antiserum.
2. From the specific activity of the labelled material, calculate the mass which may be added.
3. Determine the approximate dilution of antiserum that may be used, and confirm the ability of authentic material to displace the labelled under defined conditions.
4. Calculate the dilution of antiserum to give 60–70% binding of radioactivity, and find the pH (from 6 to 10) which gives maximum binding.
5. Determine the rate of binding of labelled steroid at various temperatures.
6. Determine the optimum dilution of antiserum, that gives maximum displacement under the conditions selected.

With regard to point 2, a defined amount of radioactivity must be added to the system in order to obtain reasonable counting times and errors. If it is assumed that the radioactive and authentic materials are treated in an identical manner in the assay, and that they are mixed prior to the addition of antibodies, then a mass of non-labelled material that is equivalent to 4 times the mass of the labelled material should, under optimum conditions, give a reduction in the initial binding of nearly 80%. These conditions rarely apply with an iodinated derivative due to the marked differences in the association constants, and the optimum conditions must be determined experimentally.

Table 2. The relative potency of various steroids to compete with tritiated oestradiol for binding sites on antibodies to oestradiol-3-succinyl-BSA (OE-3-S), oestradiol-6-carboxymethyl-oxime-BSA (OE-6-CMO) and oestradiol-17β-succinyl-BSA (OE-17β-S). DCC was used to remove the unbound steroid from systems in equilibrium

Steroid	Anti-OE-3-S	Anti-OE-6-CMO	Anti-OE-17β-S
Oestradiol-17β	100	100	100
Oestradiol-17α	<1	2	6
Oestrone	8	<1	62
Oestriol	<1	<1	18
6-Oxo-oestradiol	<1	5	<1
Oestradiol-17β-glucuronoside	—	<1	140*
Oestradiol-17β-sulphate	—	<1	140*
Oestradiol-3-glucuronoside	180*	<1	—
Oestradiol-3-sulphate	180*	<1	

* Values highly dependent upon number of immunizations.

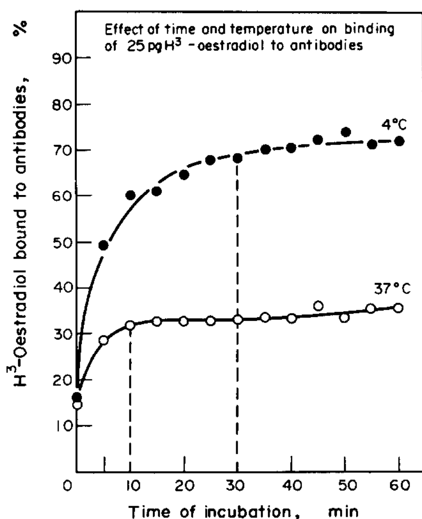

Fig. 2. The effect of time and temperature on the binding of [2,4,6,7-^{3}H]-oestradiol to antibodies against oestradiol-6-carboxymethyl oxime-BSA in the liquid phase.

Effect of pH

The optimum pH in terms of initial percentage binding and overall displacement by 100 pg of oestradiol has been determined to be approx. 8·0.

Reaction kinetics

The rate of binding of tritiated oestradiol and oestradiol-carboxymethyl oxime-mono-iodohistamine at various temperatures has been determined experimentally and the results for the tritiated material are shown in Fig. 2. In general, a constant level of binding is obtained more rapidly at higher temperatures, but the overall binding is less. In the example at 4°C, relatively constant binding at an adequate level is obtained after 30 min.

The rate of binding is similar for the iodinated derivative (maximum binding at 37°C is obtained after 30 min), but the lower dissociation constant minimises the difference in values between the two temperatures.

Corresponding experiments with anti-bodies linked to cellulose (i.e. a solid-phase system) have shown that the time to reach equilibrum is increased (1·5–4 h), but the assay may be performed at room temperature as the rate of dissociation is not so temperature dependent.

Dilution of antiserum

Finally the optimum dilution of antiserum, for the maximum displacement of labelled material, may be found by incubating labelled oestradiol and labelled oestradiol plus 50 pg of authentic oestradiol with serial dilutions of the antiserum. The results from a typical experiment with tritiated oestradiol are shown in Fig. 3. For this experiment, the time of incubation is that found from the rate of binding, and the labelled and non-labelled material are mixed before the addition of antisera. The amount of labelled material bound to the antiserum is plotted on the ordinate, against the inverse of the dilution of the antiserum on the abscissa (log scale). The upper line shows the effect of dilution on the initial binding, and the lower line, the effect in the presence of the non-labelled material. The third line is the difference, which represents the displacement.

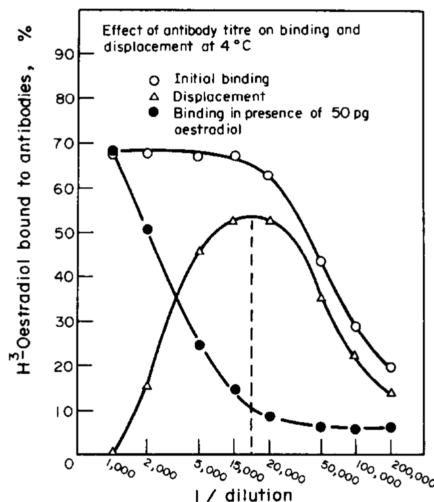

Fig. 3. The effect of antisera dilution on the initial binding of [2,4,6,7-^{3}H]-oestradiol to antibodies against oestradiol-6-carboxymethyl oxime-BSA, and the inhibition by 50 pg of authentic oestradiol.

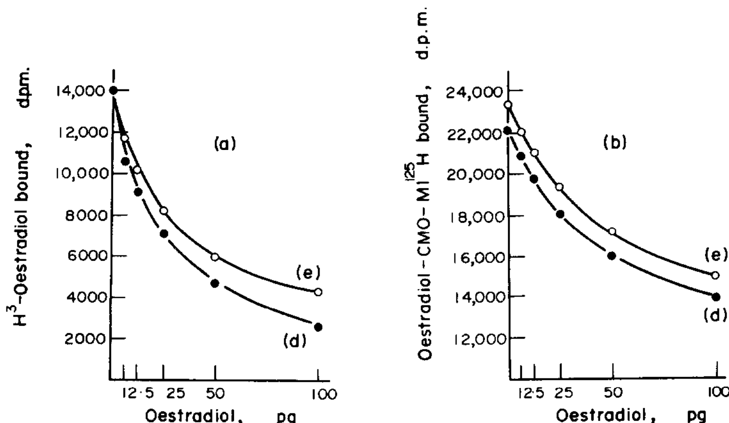

Fig. 4a and 4b. Standard curves for oestradiol using [2,4,6,7-^{3}H]-oestradiol or oestradiol-6-carboxymethyl oxime-histamine I^{125}.

Table 3. Some characteristics of reagents used in the preparation of optimized standard curves for oestradiol

Labelled tracer	CPM added	S.A. (Ci/mmol)	pg Added	Dilution of antiserum	CPM displaced by 100 pg oestradiol
2,4,6,7-[^{3}H]-oestradiol	7000	100	25	1:2000	4000
Oestradiol-6-CMO-histamine-I^{125}	20,000	7000	4	1:4000	5000

* The counting efficiencies of tritium and iodine-125 are 30 and 60% respectively.

Standard curves

A typical standard curve (E) for oestradiol produced under conditions determined by this procedure is shown in Fig. 4a. The pH of the buffer was 8·0 and the time of incubation 30 min; 20,000 d.p.m. (25 pg) of [^{3}H]-oestradiol, and an antiserum against oestradiol-6-carboxymethyl-oxime-BSA, at a dilution of 1 in 2000 (v/v) were added in sequence. The antibody-bound material was removed by the double antibody pre-precipitation technique. Under these conditions, there is approximately 8000 d.p.m. displacement over a mass range of 100 pg. It is possible to increase the sensitivity of the standard curve by a factor of 2 (depending on the characteristics of the antibodies) by using a disequilibrium technique (curve D). In this procedure the antisera is allowed to incubate with the non-labelled material for 30 min at 4°C, in order to saturate the binding sites before the addition of labelled steroid. The mixture is then allowed to equilibrate for an additional 30 min before the separation of antibody-bound and free fractions.

The corresponding curves from an assay system in which oestradiol-6-carboxymethyl-oxime-histamine-I^{125} (20,000 c.p.m./4 pg) was used is shown in Fig. 4b. With this tracer the antiserum was used at a dilution of 1 in 4000 (v/v). Curve D shows the results from the disequilibrium technique in which the labelled material was allowed to equilibrate for 30 min. A detailed comparison on the effect of the two labelled tracers on the characteristics of standard curves using the optimized, disequilibrium techniques and the pre-precipitation double antibody method is shown in Table 3.

Effect of sample preparation and assay volume

The standards and plasma extracts have been prepared in acetone or buffer and a comparison has been made of the results. If the organic solvent is used, care must be taken to ensure that interfering material is not introduced into the system. In addition, there is some apparent loss (20%) of oestradiol, when the aliquots in acetone are dried and then redissolved in the assay tube.

Methods of separating antibody-bound and free fractions

The various reagents and their final concentrations in the assay tube are listed in Table 4. The final volume with DCC, ACS and PEG was 1·3 ml and 0·5 ml with DA. The nature and proportions of the constituents were determined by a systematic series of experiments. In the procedures, in which tritiated oestradiol was used with DCC, the bound fraction was decanted, and the amount of radioactivity determined in a counting vial after the addition of 10 ml of scintillation fluid. Furthermore, in many of the experiments, the level of radioactivity in the free fraction was determined after resuspension of the carbon pellet in 300 μl of methanol, and the addition of 1 ml of scintillation fluid [27]. In the methods in which ACS, PEG or DA were used, the free fraction was decanted, and the precipitate resuspended in the assay tube with 400 μl of water prior to the addition of 1 ml of SF. When the iodinated derivative was used as tracer the amount of radioactivity in the precipitate was determined in the assay tube.

Effect on some characteristics of the standard curve

The effects of separation technique on some characteristics of the standard curve are shown in Table 5. The results show that the level of non-specific binding is relatively high if ACS is used, whereas the apparent binding to antiserum (i.e. total binding minus non-specific binding) is relatively low for PEG. The level of non-specific binding for all four methods, has been shown to be independent of the mass of steroid in the system, and it is independent of temperature between 4° and 37°C. In addition, the level remains remarkably constant between assays [31].

Effect of time

The effect of separation technique on the apparent binding of [^{3}H]-oestradiol at 4°C with respect to time is shown in Table 6. It may be seen that if DCC is used, labelled material is removed rapidly over the first 10 min (approx. 3500 d.p.m./min) For the next 10–15 min there is suggestive evidence that a plateau has

Table 4. Some reagents for the separation of antibody-bound and free steroids

Method	Final amount in assay tube
DCC (Dextran-coated charcoal)	2 mg Charcoal; 0·2 mg Dextran T70
ACS (ammonium, calcium sulphates)	50% Sat. AS; 40 mg CS
DA (double antibody)	1:200 2° Ab; ±40 mg CS
PEG (polyethylene glycol, mol. wt. 4000)	23% PEG; 40 mg CS

Table 5. The effect of separation technique on some characteristics of standard curves for oestradiol

Method	2,4,6,7-3H-Oestradiol NSB (%)	ABA (%)	D (d.p.m.)	Oestradiol-6-CMO-MIH NSB (%)	ABA (%)	D (d.p.m.)
DCC	5	66	10,600	1	51	7700
ACS	28	63	9600	30	27	3000
DA	4	65	9800	3	48	8500
PEG	6	43	7000	—	—	—

NSB is the non-specific binding, ABA the apparent binding to antibodies (i.e. total binding minus non-specific binding), D is the displacement by 100 PG of authentic oestradiol.

Table 6. The effect of various reagents on the apparent binding (d.p.m.) of tritiated oestradiol with respect to time. In the case of DA a pre-precipitation technique was used

Time (mins)	DCC	ACS	DA	PEG
0	18,600	22,000	—	9600
5	16,800	23,800	—	11,300
10	15,100	23,800	—	11,200
15	14,800	23,800	—	11,200
20	13,900	24,100	—	11,500
25	13,300	23,500	—	12,100
30	13,000	23,000	—	12,100
40	12,600	23,700	—	12,600
50	12,400	21,600	—	12,800
60	12,400	21,400	—	11,900

been reached, and this phase is followed by a progressive decrease at a reduced rate for up to 60 min. An opposite pattern is observed if PEG is used to precipitate the bound fraction, whereas the results show that ACS or DA efficiently precipitate the steroid-antibody complex with only minimal disturbance to the primary reaction.

Effect of centrifugation

The effect of centrifugation (2000 g at 4°C) for progressive periods of time has been studied. The first two samples were removed after 5 and 10 min respectively, and the remainder at 15 min intervals. The results show that there was a tendency for the apparent binding to decrease in the presence of DCC (8 d.p.m./min) and increase with PEG (15 d.p.m./min), ACS (32 d.p.m./min) and DA (51 d.p.m./min). There were no significant changes in the levels of non-specific binding (NSB).

As a result of these studies, the various reagents (DCC, PEG and ACS) were allowed to remain in contact with the primary reactants for 10 min, prior to centrifugation at 4°C for 10 min. If the double antibody pre-precipitation technique was used, the tubes were centrifuged after the appropriate reaction time.

Calculation of results

The concentration of oestradiol (S) expressed as pg/ml of plasma was calculated according to the following formula:

$$S = \left[\frac{\alpha Tx \times Cs \times Es}{Cx \times \beta Ex} - M\right]\frac{1}{V}. \quad (1)$$

Where α is the correction factor for the aliquot taken for radioimmunoassay, and Tx the reading from the standard curve; Cs is the c.p.m. in the aliquot β of the unknown; Ex and Es are the counting efficiencies of the unknown and standard and M the mass of internal standard. V is the volume in ml of plasma. If an iodinated tracer is used the calculation is simplified as there is no need to correct for quenching.

Studies have been made on the use of various equations to linearize the standard curves, or for computer fitting. The most successful of the former approaches involves a logit, log transformation:

$$\log\left(\frac{y}{1-y}\right) \textit{ vs } \log x \quad (2)$$

where y is the fraction of radioactivity bound to antibodies, and x is the mass of oestradiol added.

In the second approach, successful fitting has been achieved by making either x or y the variable function:

$$y = \frac{a + a_1x + a_2x^2}{x + a_3} \quad (3)$$

or

$$\log x = b + b_1 \log y + b_2 \log^2 y + b_3 \log^3 y \quad (4)$$

EVALUATION

Precision

An estimate of precision was obtained by the analysis of plasma samples in duplicate, and the subsequent analysis of the results according to the procedure proposed by Snedecor[32]. In this method the coefficient of variation (CV) is given by the following equation:

$$CV = \left(\frac{d^2}{2n}\right)^{1/2} \quad (5)$$

where

$$d = \left(\frac{\text{highest} - \text{lowest value of duplicates}}{\text{lowest value of duplicate}}\right) \times 100$$

and n = number of duplicate determinations.

On 40 duplicate determinations performed in the same assay, with values ranging from 30 to 300 pg/ml, the coefficient of variation using tritium or iodine-125

as tracer, and DA to separate bound and free fractions was 8%. In 20 duplicate determinations performed over four different assays, the coefficient of variation was 14%. Similar values were found on a comparative study of 12 samples using tritiated oestradiol and DCC, ACS, PEG or DA.

Sensitivity

The lower limit of oestradiol that can be determined depends upon the error associated with the measurement of this value and upon the method blank. As the standard deviation on the first point of the standard curve (3%) never overlaps the standard deviation of the corresponding O, a reading of 6·25 pg on the standard curve may be taken as the lower limit of sensitivity. This value corresponds to 18·7 pg/ml of plasma if it is assumed that an 80% recovery was obtained. The method blank (using DCC) as deduced from the values for water taken through the whole procedure in duplicate (200) over a period of 3 yr is 9 ± 6 pg/ml. Hence a value of 20 pg/ml may be taken as the limit of sensitivity of the assay under most circumstances. The corresponding blank value if ACS is used is 12 ± 4 pg/ml, 11 ± 5 for PEG and 9 ± 7 for DA. The resolving power of the method may be defined as that value which is significantly different from the next. An estimate of this value was obtained by undertaking replicate analyses of plasma samples containing different amounts of oestradiol. The standard deviation about the mean d.p.m. (^3H) or c.p.m. (^{125}I) was calculated for every sample, and the corresponding standard deviation on the mass was read from the standard curve. The mean standard deviations of the masses, corrected for aliquots and sample volume was 5 pg/ml of plasma. Accordingly, when the concentrations from different samples varied by less than twice this amount the results were not considered to be significantly different.

Specificity

The principal factor affecting the overall specificity of the method is the characteristics of the particular batch of antiserum. In this context, the specificity of the respective antisera, in terms of the ability of closely related steroids to compete with tritiated oestradiol for binding sites, has been reported for anti-oestradiol 17β-succinyl-BSA [4], anti-oestradiol-6-carboxymethyl oxime-BSA [19, 21] and anti-oestradiol-11α-succinyl-BSA [24]. In the present study essentially similar results were obtained, although there was some batch to batch variation. Accordingly an antisera to oestradiol-6-carboxymethyl oxime-BSA (donated by Professor H. R. Lindner) was selected for further study.

The choice of [2,4,6,7-^3H]-oestradiol or an iodinated derivative (oestradiol-6-carboxymethyl oxime-6-histamine I^{125}) has an effect on specificity. The slight isotopic effects observed with the tritiated material, and the increased K value associated with the iodinated complex leads to a statistically insignificant reduction in the calculated values for oestradiol in plasma. Furthermore, with many samples a slight reduction in value is obtained if a disequilibrium assay is used rather than allowing the reactants to reach equilibrium prior to the separation of antibody-bound and free fractions.

The type of separation technique consistently affects the apparent concentration of oestradiol in peripheral venous plasma, and the results of a comparative study using either DCC, ACS, DA or PEG are shown in Table 7. In this study tritiated oestradiol was used as tracer, and the values obtained by all methods in the various groups of plasma samples (at least six per group) are within the generally accepted range, but if a paired t-test is used to compare the effect of separation technique, then the values obtained on late pregnancy plasma are significantly lower ($P < 0·01$) using ACS. Similarly, the values using this reagent are significantly lower on plasma samples removed during the menstrual cycle ($P < 0·05$). The extraction procedure affects the final result and overestimates in the order of 2–6-fold have been obtained on untreated plasma, and from 4- to 19-fold on plasma that had been heated at 150°C for 15 min, in order to destroy those proteins responsible for nonspecific binding. If methylene dichloride was used in place of diethyl-ether, then both the blank value (20 ± 5 pg/ml) and the unknowns were increased (5–33%). The effect of chromatography on the final values in plasma from women is shown in Table 8. In this experiment the assay system contained tritiated oestradiol and an antiserum to oestradiol-6-carboxymethyl oxime-BSA. The antibody-bound steroid was precipitated by the addition of ACS. It may be seen that under optimised conditions the mean values are very similar. Finally, the method of preparing the standards and extracts (either in an organic solvent or buffer) does not seem to affect the final result, neither does the method of curve fitting.

Recoveries

The percentage recovery (mean ± S.D.) of tritiated oestradiol after extraction was 92 ± 6, and after the chromatographic step this value was reduced to 58 ± 12.

DISCUSSION

The results presented are of a circular study in which each variable has been evaluated in terms of the optimum conditions derived from the preceding experiment. In addition, it should be emphasized that although tritiated oestradiol or an iodinated derivative

Table 7. The effect of separation technique on the apparent concentration of oestradiol (pg/ml, mean ± S.D.) in peripheral venous plasma if tritiated oestradiol is used in the assay system

Method	Plasma source: Non-pregnant women	Plasma source: Pregnant women
DCC	126 ± 60	14,600 ± 3700
ACS	83 ± 57	11,900 ± 4000
DA	112 ± 59	12,300 ± 3900
PEG	129 ± 83	16,300 ± 3700

Table 8. The effect of chromatrography on the apparent concentration of oestradiol (pg/ml; mean ± S.D.) in peripheral venous plasma

Plasma source	No. of determinations	Before chromatography	After chromatography
Men			
(aged 18–40 yr)	12	21 ± 4	19 ± 5
Women			
(days 1–10 of menstrual cycle)	20	40 ± 33	39 ± 29
(Days 11–17)	8	140 ± 80	142 ± 86
(Days 18–32)	20	62 ± 34	61 ± 37
12th–41st week of pregnancy	20	11,900 ± 4000	14,900 ± 3500

can be obtained in a reasonably radioimmunologically pure form, no two batches of antisera are exactly the same—even to the same antigen. However, notwithstanding these limitations some general conclusions may be derived.

For example, it is apparent that under all conditions antisera to oestradiol-6-carboxymethyl oxime-carrier molecule, are the most useful in terms of specificity. Furthermore, a mixture of ammonium calcium sulphates or the pre-precipitation double antibody technique would appear to be the methods of choice for the separation of antibody-bound and free fractions. These techniques provide the most accurate results with minimum disturbance to the primary reaction. The reagents are easy to use, and the appropriate bound fraction is rapidly separated and may be counted in the assay tube. The principal disadvantage to the use of ammonium calcium sulphates is the relatively high amount of non-specific binding, but this parameter is independent of the total mass of steroid in the system. A disadvantage to the large scale application of the double antibody method is that the reagent is relatively expensive.

At present, the choice of labelled tracer is between tritium and iodine-125. In chemical terms the relative merits may rest with the particular batch of antiserum, and the structure of the iodine containing derivative. The sensitivity, precision and specificity of the method will depend upon the relative association constants of these two labelled substances and authentic oestradiol for binding sites on the population of antibodies. The advantages and disadvantages with regard to cost depend entirely upon the number of assays performed, and whether or not the radioactivity (β or γ) is determined in the assay tube. The larger the number of assays performed then the better value for money is obtained with the iodinated derivative. However, if a smaller number of assays are required at irregular intervals then the longer half-life and stability of the tritiated oestradiol becomes increasingly important. Furthermore, it should be emphasized that if oestradiol-6-carboxymethyl oxime-mono-iodohistamine is chosen as the labelled tracer, then care should be taken to check the purity of the derivative. At least nine products can arise from the primary reactions, and of those at least five may be radioactive and five immunoreactive. A major advance in the procedure would be an improvement in the yield of labelled derivative (at present approximately 4% with reference to I^{125}), and a reduction in the amount of hapten (at present 1 mg) required for activation prior to the addition of labelled histamine.

A further factor to arise from this study is the importance of optimising the assay system. The results show that the sensitivity of the standard curve can (under certain circumstances) be increased by up to a factor of 2 if a disequilibrium technique is used. In addition, there was a tendency to obtain lower results if an iodinated derivative was used in an homologous position to the antigen; and if a disequilibrium technique was employed followed by the addition of ACS to separate the bound fraction which was counted in the assay tube. With regard to the overall procedure the results show that an extraction procedure is necessary, but a chromatographic step is not required routinely for the determination of oestradiol in peripheral venous plasma from healthy men and women.

Finally, the results confirm that attempts should be made to purify the antiserum, in order that the values obtained by alternative techniques may be rationalized.

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