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COMPARISON OF QUICK METHODS FOR THE ESTIMATION OF ESTRADIOL IN PLASMA BY RADIOIMMUNOASSAY

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SUMMARY

Three methods for the determination by radioimmunoassay (RIA) of estradiol in plasma are described. Method A involves RIA, using the estradiol-17-hemisuccinate (E_2-17HS) antibody on a plasma extract purified by Sephadex LH-20 microcolumn chromatography; it permits the precise, accurate and specific determination of estradiol (E_2) and estrone (E_1) in male and female plasma. Method B involves RIA with a 6-keto-estradiol (6-keto-E_2) antibody on a crude plasma extract. It is rapid, precise and accurate and allows the relative specific determination of estradiol in female plasma during the menstrual cycle; it is not specific for estradiol in male plasma. In method C, plasma estradiol is measured in plasma without prior extraction with the 6-keto-E_2 antibody: it is not specific for estradiol, but the value obtained is a valid parameter of the estradiol concentration in pregnancy plasma. In view of its simplicity and its relative specificity as shown by comparison of the results with methods of known specificity, method B can be recommended for the rapid monitoring of plasma estradiol levels both during the menstrual cycle and in pregnancy.

Since the first description of a radioimmunoassay (RIA) for the estimation of plasma estradiol by Abraham [1], several RIA methods for the determination of estradiol and estrone after purification of the plasma extract by either t.l.c. [2, 4], LH-20 [5–12], celite column chromatography [13], or liquid–liquid partition [14] have been published. The recent introduction of more specific antibodies [15–17] however has opened the perspective of the direct measurement of estradiol in crude plasma extracts [9, 18] or even in unextracted plasma. We have compared the results obtained by such quick methods with those obtained by a reliable, but somewhat more elaborate, method permitting the separate determination of estrone (E_1) and estradiol (E_2).

MATERIALS AND METHODS

Antisera

The antiserum to 17β-estradiol hemisuccinate bovine serum albumin (E_2-17-HS) was a gift from Dr. Abraham (Harber General Hospital, University of California, Torrance). The antiserum to estradiol-6-(O-carboxymethyl)-oxime bovine serum albumin (6-keto-E_2 antibody) was a gift from Dr. Furr (Department of Physiology and Biochemistry, University of Whiteknights, Reading). In later experiments an antibody obtained in our laboratory by immunization of rabbits with estradiol-6-(O-carboxymethyl)oxime-BSA, synthesized according to Dean *et al.* [19] was used; this antibody and the antibody obtained from Dr. Furr had similar specificity.

The E_2-17-HS antibody was diluted with phosphate buffer at a titer of 1:90·000 for the determination of E_2, and of 1:60·000 for the determination of E_1; the 6-keto-E_2 antibody was used at a dilution 1:25·000.

After dilution 1 μCi of [2,4,6,7-^{3}H]-estradiol respectively estrone, were added to 20 ml of antiserum.

Radioactive steroids

[2,4,6,7-^{3}H]-estrone, S.A. 116 Ci/mmol and [2,4,6,7-^{3}H]-estradiol, S.A. 100 Ci/mmol were obtained from the Radiochemical Centre, Amersham, England. Reference steroids and standards were obtained from Steraloids (Pawling, N.Y.). Isatine (2,3-indolinedione) and 1,4-diaminoantroquinone were obtained from Koch-Light Laboratories (England). Sephadex, LH-20 and Dextran-T80 were obtained from Pharmacia, Uppsala. Charcoal was Merck No. 2186. Phosphate buffer is prepared by mixing 305 ml 0·2 M Na_2HPO_4–$12H_2O$ and 195 ml 0·2 M NaH_2PO_4–H_2O; after addition of 9 g NaCl and 1 g NaN_3, the solution is made up to 1 liter by addition of deionized distilled water. 1 g of gelatine is dissolved in the buffer at 50°C, and the buffer is stored at 4°C.

Dextran coated charcoal suspension

Equal volumes of phosphate buffer, containing respectively 1·25 g/100 ml charcoal and 0·125 g/100 ml Dextran T-80 are mixed. The suspension is stored at 4°C. During use, the suspension is continuously magnetically stirred.

Solvents

All solvents were reagent grade and used without further purification, with the exception of the ethyl ether which was purified by passing through an Al_2O_3 column (Woelm basic Al_2O_3) prior to use.

Radioactivity measurements

All counting was performed in a Packard Tricarb model 2420 using as a scintillator 10 ml of a solution containing 0·2 g POPOP, 5 g PPO and 100 g naphthalene made up to 1 liter in dioxane.

TECHNIQUES USED FOR THE DETERMINATION OF ESTRADIOL (AND ESTRONE) IN PLASMA

Method A

This method is a modification of the method of Emment *et al.* [7]. To 1 ml of female (in the postmenopausal women 4 ml may be necessary) or 2 ml of male plasma and to 1–2 ml of water (method blank), are added 2000 d.p.m. of respectively [2,4,6,7-^{3}H]-estradiol and [2,4,6,7-^{3}H]-estrone as internal standards. After mixing, the plasma is extracted with 4 ml of ether and, after freezing the plasma, the ether phase is decanted and evaporated to dryness. The residue is taken up in 100 μl of benzene–methanol (85:15, v/v) containing as indicator isatine and the pink component of 2,4-diaminoanthraquinone [20]. The extract is brought onto a Sephadex LH-20 microcolumn (Pasteur-pipette i.d. 7 mm; height of column = 8 cm) prepared in benzene–methanol (85:15, v/v); elution is performed with the same solvent.

The pink coloured fraction (1–1·5 ml) containing E_1 and the yellow coloured eluate, containing E_2, are collected separately. The E_1 and E_2 fractions are evaporated to dryness and taken up in 200 μl of ethanol containing 0·5% propylene glycol. After washing the column with 5 ml of benzene–methanol (50:50, v/v) and equilibration with benzene–methanol (85:15), the column may be re-utilized for several months.

Assay procedure

An aliquot of the extract (usually 50 μl) is taken for recovery determination, whereas 100 μl are evaporated in a small disposable glass tube (10 × 75 mm) for RIA.

A series of standard (0, 10, 20, 50, 100 and 200 pg) of respectively E_1 and E_2 are prepared in triplicate and to each standard a simulated column eluate is added and evaporated to dryness.

Subsequently 0·2 ml of E_2-17HS antiserum containing ±20·000 d.p.m. of either [2,4,6,7-^{3}H]-estradiol or estrone are added to each tube and after mixing with a Vortex mixer, the tubes are incubated overnight at 4°C.

Separation of the bound from free steroid is performed by adding 1 ml of a cooled charcoal–dextran suspension. After standing for 20 min at 4°C, the tubes are centrifuged at 3500 ***g*** for 5 min, the supernatant (bound fraction) is carefully decanted in a counting vial containing 10 ml of scintillation fluid and counted to a relative S.E. of less than 2%.

The standard curve is constructed using a desk-top computer, plotting the reciprocal of the fraction bound (Bo/B) against the concentration, Bo representing the binding in the zero standard. Computed standards were always compared to actual values and if a difference of more than 5% was observed, the highest value of the standard curve was discarded; if the computed values still differed by more than 5% from the real value (this occurred so far only once) the complete assay was discarded.

Method B

After addition of 2000 d.p.m. of [2,4,6,7-^{3}H]-estradiol as an internal standard, the plasma is extracted with 4 ml ether. An aliquot of the extract is taken for recovery whereas RIA is performed on another aliquot as described for method A, using however the 6-keto-E_2 antibody. To the standard tubes an equivalent volume of ether is added and evaporated to dryness.

Method C

Radioimmunoassay is performed directly on 2–5 μl of pregnancy plasma and the incubation with the 6-keto-E_2 antibody is reduced to 1 h at 4°C. Separation of the bound from the free fraction is performed as usual, using charcoal–Dextran.

RESULTS

A. *Reliability criteria for method A*

The specificity of the 17-HS-E_2 antibody was studied by determining the relative affinity for different steroids at 50% binding (Table 1). It can be seen that besides for estradiol, this antibody has a *high* affinity for estrone, 17α-estradiol, 16-keto-estradiol, and for estriol. Moreover it exhibits *moderate* affinity for 2-methoxy-estrone, 6α-hydroxy- and 6-keto-estradiol as well as for 2-methoxy-estradiol, for 17- and 16-epies-

Table 1. Specificity of antibodies: relative affinity of steroids for the antibodies at 50% binding

	E_2-17HS	6-keto-E_2	Occurrence in plasma [Reference]
1,3,5(10)-estratriene-3,17β-diol (E_2)	100	100	
1,3,5(10) estratriene-3,17α-diol (17α-E_2)	21	1	
3,17β-dihydroxy-1,3,5(10)-estratrien-6-one (6-keto-E_2)	6	81	
1,3,5(10)-estratriene-3,6α,17β-triol (6α-hydroxy-E_2)	3	47	
3,17β-dihydroxy-1,3,5(10)-estratrien-16-one (16-keto-E_2)	10	8	+ + in P* [21]
2,3,17β-trihydroxy-1,3,5(10)-estratriene-2-methylether (2-methoxy-E_2)	5	5	
3-hydroxy-1,3,5(10)-estratrien-17-one (E_1)	56	4	
2,3-dihydroxy-1,3,5(10)-estratrien-17-one (2-hydroxy-E_1)	1	1	+ + + [22]
2,3-dihydroxy-1,3,5(10)-estratrien-17-one-2-methylether (2-methoxy-estrone)	1	1	+ in P [21]
3,16α-dihydroxy-1,3,5(10)-estratrien-17-one (16α-hydroxy-estrone)	1	1	+ in P [21]
1,3,5(10)-estratriene-3,16α,17β-triol (E_3)	9	7	
1,3,5(10)-estratriene-3,16α,17α-triol (17-epi-E_3)	3	2	− [21]
1,3,5(10)-estratriene-3,16β,17β-triol (16-epi-E_3)	5	8	+ in P [21]
1,3,5(10)-estratriene-3,15α,16α,17β-tetrol (15α-hydroxy-E_3)	3	3	+ in P [23, 24]
1,3,5(10)-estratriene-2,3,16α,17β-tetrol (2-hydroxy-E_3)	4	4	
17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol (ethynylestradiol)	5	5	
17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol-3-methylether (Mestranol)	1	1	
17β-hydroxy-4-androsten-3-one (testosterone)	<0·01	<0·01	
4-Androstene-3,17-dione	<0·01	<0·01	
3β-hydroxy-5-androsten-17-one (DHEA)	<0·01	<0·01	
4-pregnene-3,20-dione (progesterone)	<0·01	<0·01	
17-hydroxy-4-pregnene-3,20-dione (17OH-progesterone)	<0·01	<0·01	
17β-hydroxy-5α-androstan-3-one (dihydrotestosterone)	<0·01	<0·01	
1,3,5(10)-estratriene-3,17β-diol-17-glucuronide	55	63	
1,3,5(10)-estratriene-3,17β-diol-3-glucuronide	1	7	
1,3,5(10)-estratriene-3,17β-diol-3,17-diglucuronide	1	11	
3-hydroxy-1,3,5(10)-estratrien-17-one-3-glucuronide	1	1	
1,3,5(10)-1,3,5(10)-estratriene-3,17β-diol-17-sulfate	52	4	
1,3,5(10)-1,3,5(10)-estratriene-3,17β-diol-3-sulfate	2	16	

* P: pregnancy.

triol, as well as for 2-hydroxy- and 15α-hydroxy-estriol.

However, only a few of these metabolites occur in human plasma. Indeed, 17α-estradiol and 17-epi-estriol [21], 6-keto- and 6α-hydroxy-estradiol and 2-hydroxy-estriol do not occur in any significant concentration in human plasma; 16α-hydroxy-estrone and 16-epiestriol are found in a concentration of 2 ± 2 ng/ml in term pregnancy plasma [21]; 16-keto-estradiol has been found in a concentration of 2·3 ± 1·9 ng/ml and 2-methoxy-estrone in a concentration of 1·5 ± 1·4 ng/ml [21] in a term pregnancy plasma, whereas 2-hydroxy-estrone which has however a low affinity for both antibodies, occurs in female and male plasma in a concentration similar to the concentration of estrone, except during pregnancy where the concentration varies between 1·2 and 3·6 ng/ml [24]. Fifteen hydroxy-estriol finally occurs during pregnancy in a concentration of about 10% of the estradiol concentration [23–24].

The E_2-17-HS antibody cross reacts also strongly with 17-mono-conjugates of estradiol (Table 1) but these are not extracted by ether [25].

Taking into account the cross-reactivity of the steroids with the E_2-17 HS antibody on the one hand, and their concentration relative to estradiol in pregnancy plasma on the other hand (Table 2), it is apparent that none of these steroids will interfere to any significant extent with either the E_1 or E_2 determination. Further evidence for the specificity of the method may be deduced from the values obtained for the blanks: the water blank both for estrone and estradiol is negligible (0 ± 0·5 pg) whereas the plasma blank obtained with charcoal treated plasma, varied between 0 and 2 pg per sample.

Table 2. Interference of plasma estrogen with the determination of E_2 and E_1

Steroid	Approximate concentration relative to E_2 (pregn. plasma)	Reference	Affinity for AB E_2-17HS (% of E_2)	Affinity for AB 6-keto-E_2 (% of E_2)	Product relative conc. and affinity E_2-17HS	Product relative conc. and affinity 6-keto-E_2
2-methoxy-estrone	±10–20%	21	1	1	0·001	0·001
2-hydroxy-estrone	10–20%	22	1	1	0·001	0·001
15α-hydroxy-estrone	trace	21	?	?	trace	trace
16α-hydroxy-estrone	15–30%	21	1	1	0·003	0·003
16-ketoestradiol	15–30%	21	10	8	0·01–0·03	0·01–0·03
16-epi-estriol	10–20%	21	5	8	0·005–0·01	0·01–0·02
17-epi-estriol	0	21	3	2	0	0
estriol	50%		9	7		
2-hydroxy-estriol	0		4	4	0	0
15-hydroxy-estriol	10%	23–24	3	3	0·03	0·03

In order to evaluate the influence of different plasma volumes on the estrogen concentration, results obtained when extracting one, respectively 2 ml of female plasma and 2 respectively 4 ml of male plasma were compared. As can be seen from Table 3, values obtained for both estrone and estradiol were independent of the volume extracted. This implies that the dose response curve with the plasma extract is parallel to the curve obtained with the standards and is additional evidence for the specificity of the method.

Precision was evaluated by determining intra- and interassay variability.

The precision of the standard curve in the range 10 to 200 pg varied between 2 and 5 pg; at 10 pg the coefficient of variation was 27%.

The intraassay variability in male plasma (n = 10) was 11·3% at a concentration of 2·2 ng/100 ml for estradiol and 9·9% at a mean concentration of 4·5 ng/100 ml for estrone; for female plasma the coefficient of variation was 12·5% at a concentration of 4·8 ng/100 ml for estradiol, and 12·2% at a concentration of 7·2 ng/100 ml for estrone. The interassay variability was 10·7% for estradiol and 7·8% for estrone in males (five different pools of different concentration measured at 5 consecutive days), 10·4% for estradiol and 10·3% for estrone during the female menstrual cycle (six pools measured at 5 different days).

The accuracy of method A was determined from recovery experiments in which 100 and 200 pg of estradiol, respectively estrone, were added to the plasma sample. Recovery for estradiol was 92·4 ± 11·2 (S.D.)% at 100 pg and 108 ± 11·5 (S.D.)% at 200 pg (n = 9) and 106 ± 13·7 (S.D.)% at 200 pg (n = 9) of estrone.

As far as *practicability* is concerned one technician can make 50 plasma determinations in 2 days. *Normal*

Table 3. Influence of different plasma volumes on estrone and estradiol values measured (method A)

Plasma	E_1 (ng/100 ml)		E_2 (ng/100 ml)	
Female				
Volume extracted	1 ml	2 ml	1 ml	2 ml
Plasma:				
A	7·3	6·3	3·8	4·4
B	6·4	6·7	3·7	4·0
C	7·2	6·8	3·6	3·7
Male				
Volume extracted	2 ml	4 ml	2 ml	4 ml
Plasma:				
D	4·4	4·4	1·9	2·2
E	4·7	4·9	2·2	2·2
F	4·4	4·7	2·8	2·4
G	3·0	2·6	5·7	5·9

Table 4. Normal values for plasma estrone and estradiol obtained with method A

	E_1 (ng/100 ml)	E_2 (ng/100 ml)
Males 20–50 yr (n = 20)	4·3 ± 0·6 (S.D.)	1·4 ± 0·2 (S.D.)
Postmenopausal women (n = 20)	4·0 ± 1·6 (S.D.)	1·1 ± 0·5 (S.D.)
Fertile females (n = 11)		
Early follicular phase	5·8 ± 1·6 (S.D.)	3·6 ± 1·6 (S.D.)
Estradiol peak	15·4 ± 3·4 (S.D.)	19·9 ± 3·3 (S.D.)
Mid luteal phase	11·4 ± 3·9 (S.D.)	11·1 ± 3·4 (S.D.)
	E_1 (ng/ml)	E_2 (ng/ml)
Pregnancy		
5–10 weeks (n = 31)	0·58 (0·11–3·0)*	0·71 (0·13–3·98)
10–20 weeks (n = 134)	2·62 (0·80–8·59)	3·95 (1·41–11·12)
20–30 weeks (n = 97)	8·74 (3·92–19·52)	12·72 (6·51–24·84)
30–40 weeks (n = 135)	12·00 (5·04–28·56)	17·89 (9·90–32·35)

* 95% confidence limits.

values for estrone and estradiol obtained with this method are given in Table 4.

B. *Reliability criteria for method B*

The specificity of the 6-keto-estradiol antibody is shown in Table 1. As expected, 6α-hydroxy- and 6-keto-estradiol show a high affinity for the antibody. However these steroids do not occur to any measurable extent in plasma. Many other estrogens show an affinity for the antibody, equivalent to a few per cent of the affinity of estradiol, but of these only estrone, 2-hydroxy-estrone, 16-keto-estradiol, estriol and 15α-hydroxy-estriol occur in a relatively high concentration in human plasma. 2-hydroxyestrone however will not interfere significantly, as its relative affinity is only 1%; 15α-hydroxy-estriol, occurring in pregnancy plasma at a concentration of about 10% of estradiol [23, 24] would not interfere either, as its relative affinity is only 3%. It seems therefore that in a crude plasma extract only estrone and estriol, and in pregnancy also 16-keto-estradiol [21], would interfere with the estradiol determination.

Taking into account their concentration in plasma, it may be estimated that theoretically the estradiol metabolites would increase the apparent E_2 concentration during the cycle by about 15%; during pregnancy the interference would be less as estriol levels are much lower than estradiol levels [18, 25], whereas 16-keto-estradiol levels are too low [21] to interfere significantly.

The plasma *blank* (3 ± 1 pg) was slightly higher than in method A.

When one, respectively 2 ml of female plasma were extracted, there was no statistically significant difference between the results obtained.

The intraassay variability, an index of the *precision*, using plasma from the menstrual cycle was 9·3% at 5·7 ng/100 ml, 7·9% at 23 ng/100 ml (n = 10), the interassay variability respectively 10·2 and 8·9% (n = 10).

The sensitivity, estimated as the 95% confidence limit of zero dose, is 12 pg per sample of plasma.

The recovery, an index of the accuracy, was 107% (n = 12) with a coefficient of variation of 10·2% for 50 pg of E_2 and 102·8% with a coefficient of variation of 12·4% for 100 pg of E_2.

C. *Comparison of results obtained respectively with method A and with method B*

(1) Female plasma. When plasma estradiol levels in normal menstruating women were determined by both methods, there was an excellent correlation (r = 0·96), the regression line corresponding to the equation: $Y = 0{\cdot}716X - 0{\cdot}16$ (Fig. 1) where Y = values obtained with the 17-E_2-HS antibody.

(2) Male plasma. The correlation between results for estradiol obtained by both methods in male plasma was poor (r = 0·53) (Fig. 2), values obtained with the rapid 6-keto-E_2 antibody method (B) being much higher than those obtained with method A. Moreover when increasing the plasma volume extracted, the estradiol values obtained with method B showed a significant decrease from linearity, indicating non parallelism between the plasma extract and the standard curve.

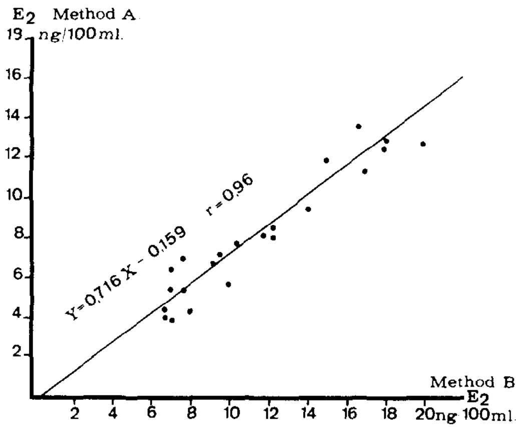

Fig. 1. Comparison of plasma estradiol levels obtained with method A, respectively method B in the female during the menstrual cycle.

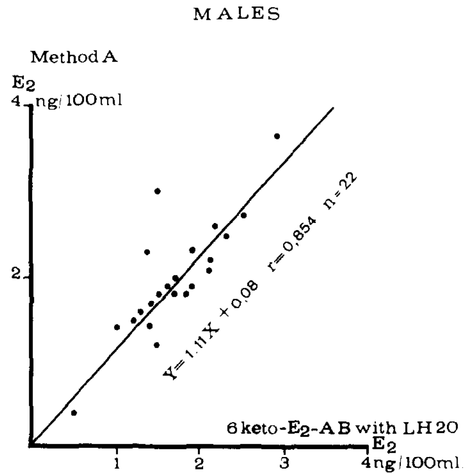

Fig. 3. Comparison of plasma estradiol levels obtained with E_2-17HS antibody, (method A) respectively the 6-keto-E_2 antibody after purification by chromatography on a LH-20 microcolumn.

When prior to the RIA with the 6-keto-E_2 antibody, the extract was purified by LH-20 chromatography, (results were however practically identical to those obtained with the E_2-17HS antibody) the regression line going almost through the origin (r = 0·854) (Fig. 3). Moreover, increasing the plasma volume did not affect the results.

(3) Pregnancy plasma (Fig. 4). The correlation between results obtained with method A and the rapid method B is excellent (r = 0·944) and, as indicated by the regression line (Y_A = 0·98 X −0·014) values obtained by both methods are almost identical. Additional purification by LH-20 column, prior to RIA with the 6-keto-E_2 antibody gives almost identical values as obtained by RIA on the crude extract. This is perhaps rather surprising as one might expect that in pregnancy some estrogen metabolites might displace estradiol from its binding sites on the 6-keto-E_2 antibody. This however apparently does not occur to any measurable extent.

D. *Evaluation of the rapid screening method for the determination of "estradiol" in late (>10 weeks) pregnancy (Method C)*

Of all the estrogens occurring in pregnancy plasma, the 6-keto-E_2 antibody reacts essentially with estradiol and its glucuronide conjugates. We investigated whether "estradiol" values obtained by RIA performed directly on pregnancy plasma would parallel the true free estradiol concentration.

The *intra-assay variability*, an index of the precision of the method, determined from the coefficient of variation of replicate (n = 5) determinations was 6% and

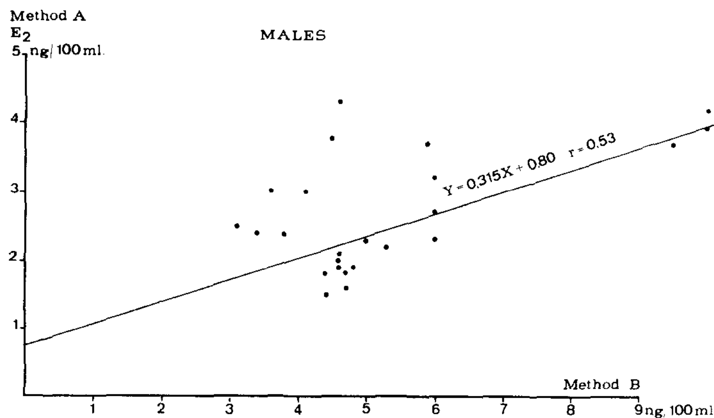

Fig. 2. Comparison of plasma estradiol levels obtained with method A, respectively method B in the male.

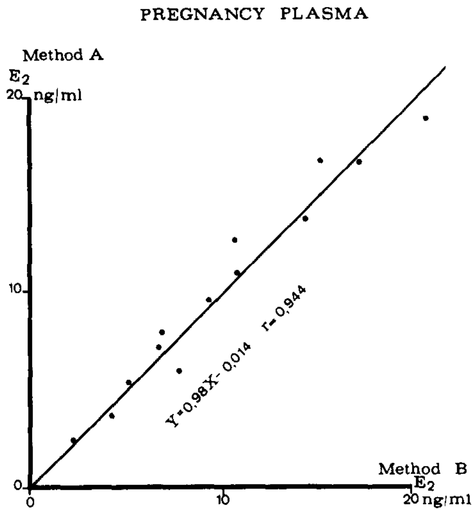

Fig. 4. Comparison of plasma estradiol levels obtained with method A, respectively method B in pregnancy.

did not vary with the concentration of estradiol (late pregnancy plasma).

Recovery experiments gave a mean recovery of 103·5 ± 10% (S.D.) when 20 pg of estradiol were added to 2·5 μl of plasma, and 99·8 ± 6·6% when 50 pg were added (n = 10). As far as the influence of the short incubation time (1 h) is concerned, it appeared that 24 h incubation yielded consistently slightly higher values, the mean difference at concentrations below 10 ng/ml being 0·13 ± 0·28 (S.E.) ng, 0·16 ± 0·33 (S.E.) ng in the concentration range between 10 and 15 ng and 1·6 ± 0·42 (S.E.) ng in the range between 15 and 25 ng. As far as the *specificity* of method C is concerned, it is evident that it is limited by the specificity of the antibody.

In order to evaluate the clinical usefulness of this method the results were compared with those obtained with method A. As can be seen on Fig. 5, the values obtained with method C are higher than those obtained with method A by about 3 ng/ml (regression line Y = 0·975 X − 3·16) but the correlation coefficient is high (r = 0·90). Moreover when estradiol values were determined sequentially during pregnancy, values obtained with either method showed a parallel evolution (Fig. 6).

When results obtained using respectively 5, 10 and

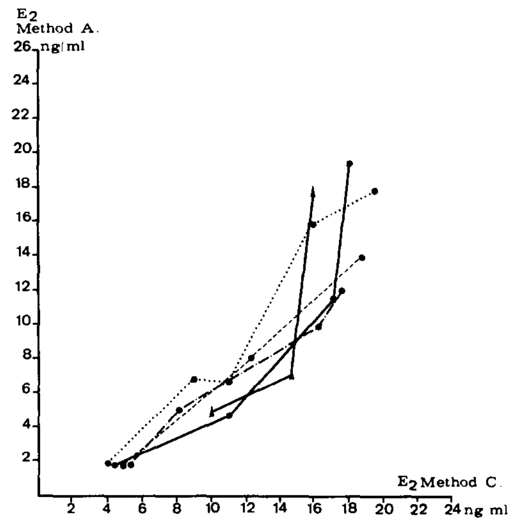

Fig. 6. Evolution of plasma estradiol levels during pregnancy; comparison of results obtained by method A and method C.

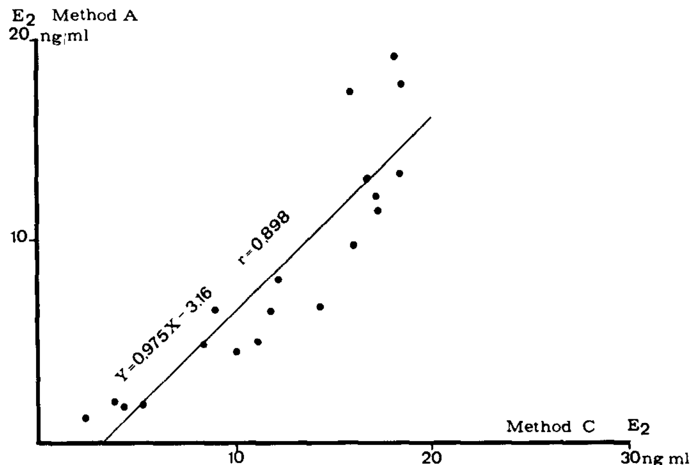

Fig. 5. Comparison of plasma estradiol levels in pregnancy obtained with method A, respectively method C.

20 μl plasma were compared it was observed that the 10 and 20 μl values were respectively 82.5 ± 1.6 and $84.7 \pm 3.1\%$ of the values obtained using 5 μl plasma (n = 22).

As it was considered that this might be attributable to the presence of high levels of sex hormone binding globulin in pregnancy plasma, the experiment was repeated after destroying TeBG at high temperature, but results remained uninfluenced. The addition of an equivalent volume of steroidfree plasma to the standard curve did not correct either for this deviation from linearity.

DISCUSSION

It is evident from our results that method A permits an accurate, precise, specific and relatively rapid determination of E_2 and E_1 in both female and male plasma. The method is very similar to the method published by Emment *et al.* [7] except for the addition of a simulated column eluate to the standards which, as shown by Cameron and Jones [9] compensates for interference caused by eluate residues and consequently decreases significantly the blank and improves the sensitivity.

Method B is rapid, precise and accurate but less specific than the more elaborate method A. The values obtained with this method during the menstrual cycle are somewhat higher than anticipated from what is known concerning the concentration of estradiol

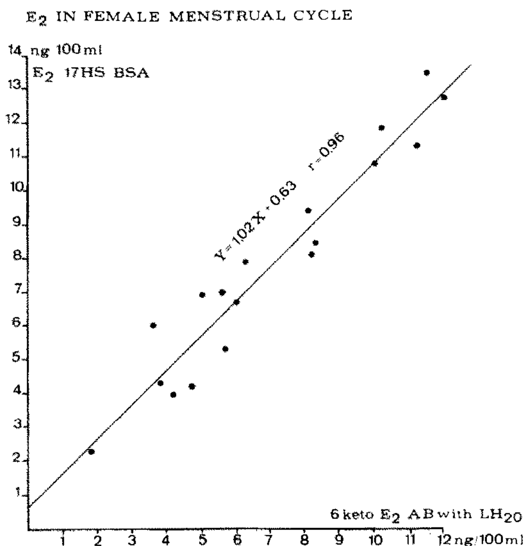

Fig. 7. Comparison of estradiol levels in female plasma obtained with the E_2-17HS antibody respectively the 6-keto-E_2 antibody on an extract purified by LH-20 chromatography.

metabolites in plasma and their affinity for the 6-keto-E_2 antibody. It should however be realized that the relative affinity in Table 1 is given at 50% binding and that at the concentration occurring in plasma the relative affinities may be different. Nevertheless, in view of the excellent correlation with the results obtained by the more specific method A, it appears that for clinical use, values obtained by this method during the menstrual cycle are a useful parameter of the true estradiol concentration.

For research work however, when an exact estimate of estradiol is required, method B cannot be recommended. Purification of the extract by LH-20 chromatography followed by RIA using the 6-keto-E_2 antibody, yields values which are slightly lower than those obtained with method A (Fig. 7). The most plausible explanation for this slight difference is the presence in the estradiol eluate of trace amounts of steroids that react with the E_2-17-HS antibody but not with the 6-keto-E_2 antibody. It seems therefore that the RIA of estradiol with the 6-keto-E_2 antibody after LH-20 chromatography of the extracts gives the most exact results.

Cameron and Jones [9] studying RIA of estradiol on a crude plasma extract with a 6-keto-E_2-antibody concluded that although precise, the method was not accurate. The reason for this lack of accuracy is not clear as the use of an internal standard should compensate for any losses due to manipulations; probably this is related to the antibody used, as under identical conditions the use of the 6-keto-E_2-antibody gave a better accuracy than the E_2-17HS-antibody used by these authors.

We found method B to be both precise, sensitive and accurate although not entirely specific. Method B cannot be used however for determining plasma estradiol in male plasma as the values obtained are too high, whereas the correlation with results obtained with method A is very poor (r = 0.57): a purification step (Sephadex LH-20 column) has to be included in the procedure in order to obtain a valid estimate. The interfering material in male plasma is unknown; testosterone can be excluded as 6000 pg of testosterone had only a binding affinity equivalent to 5 pg of estradiol.

Recently it has been suggested that plasma estradiol levels might be a good index of fetal well being [27, 28]: therefore it might be of interest to be able to follow sequentially plasma estradiol levels in pregnancy by a simple and quick method. This purpose is practically achieved by method B which allows the rapid, accurate and precise determination of estradiol in pregnancy plasma. The direct estimation of estradiol in unextracted plasma however, would represent the ultimate simplification.

Our results show that such a procedure (method C)

gives a value which is a good parameter of the estradiol concentration in plasma ($r = 0{\cdot}90$), but does not measure specifically estradiol, as is shown by comparison of the results with more specific methods and by the deviation from linearity when increasing volumes of plasma are measured. This is not unexpected in view of the presence in pregnancy plasma of several estrogen metabolites and conjugates with different affinity for the antibody. Ahmed and Kellie [29] as well as Adlercreutz and Luukkainen [21] have shown that estradiol conjugates remain at all times low during pregnancy: it is not surprising therefore that notwithstanding the interference of estradiol glucuronides, the values obtained with method C correlate well with values obtained with the more specific methods. Although this method could be used for obtaining rapidly an estimate of the estradiol concentration in pregnancy plasma, it is evident nevertheless that in view of the specificity of method B in pregnancy plasma, the latter is to be recommended for the rapid monitoring of plasma estradiol levels.

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The systematic names of steroids used in this paper are as follows:

Estradiol	1,3,5(10)-estratriene-3,17β-diol
Estradiol-17α	1,3,5(10)-estratriene-3,17α-diol
2-hydroxyestradiol	1,3,5(10)-estratriene-2,3,17β-triol
2-methodoxyestradiol	1,3,5(10)-estratriene-2,3,17β-triol-2-methylether
6α-hydroxyestradiol	1,3,5(10)-estratriene-3,6α,17β-triol
6-ketoestradiol	3,17β-dihydroxy-1,3,5(10)-estratrien-6-one
16-ketoestradiol	3,17β-dihydroxy-1,3,5(10)-estratrien-16-one
Estrone	3,hydroxy-1,3,5(10)-estratrien-17-one
2-hydroxyestrone	2,3-dihydroxy-1,3,5(10)-estratrien-17-one
2-methoxyestrone	2,3-dihydroxy-1,3,5(10)-estratrien-17-one-2-methylether
16α-hydroxyestrone	3,16α-dihydroxy-1,3,5(10)-estratrien-17-one
Estriol	1,3,5(10)-estratriene-3,16α,17β-triol
16-epiestriol	1,3,5(10)-estratriene-3,16β,17β-triol
17-epiestriol	1,3,5(10)-estratriene-3,16α,17α-triol
15α-hydroxyestriol	1,3,5(10)-estratriene-3,15α,16α,17β-tetrol