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A DIRECT RADIOIMMUNOASSAY FOR AN ESTRONE AND ESTRADIOL-17β FRACTION IN HUMAN URINE

M. CASTANIER, J. GRENIER and R. SCHOLLER
Fondation de Recherche en Hormonologie, 26 Boulevard Brune, 75 Paris XIV°

and

67–77 Boulevard Pasteur, 94 Fresnes, France

SUMMARY

A radioimmunoassay (R.I.A.) has been developed, using an antiserum that reacts with estrone and estradiol-17β. The main urine conjugates tested do not cross react significantly. Prior to the radioimmunoassay, urine aliquots are hydrolyzed by Helix pomatia digestive juice. Solvent extraction and purification steps are omitted. Toluene is used directly to separate free and bound ligands. An analysis is made of some factors influencing replication of results. Correlation with routine colorimetric and fluorimetric methods has been studied, and specificity assessed by a double isotope derivative method.

This technique, used during a Dexamethasone-Human Chorionic Gonadotropin (DXM-HCG) test in man, revealed a marked increase on the second day after HCG injection; and during a normal menstrual cycle, showed a midcycle peak.

INTRODUCTION

MOST of the described radioimmunoassays (R.I.A.) of estrogens have been applied to estimations of these compounds, in their free forms, in plasma[1–8]. Such works were attempting to improve the methodological sensitivity required by the plasma low levels of estrogens, and to achieve an estimation, as specific as possible, of a particular compound. But, then, separation procedures must be used, which themselves lengthen and complicate the method, and most often, introduce impurities from solvents and chromatographic material, thus impairing the quality of the assay.

Gurpide *et al.*[9] are not faced with such problems: they are performing a "group" estimation of estrogens in pregnancy urine, without prior hydrolysis and extraction, using a rather unspecific antiserum reacting with estriol but also with a complex fraction including other free and conjugated steroids.

In the course of our studies on plasma estrogens, a radioimmunoassay was developed, with an antiserum claimed to react mainly with estradiol-17β and estrone; the validity of the results was checked with a double isotope derivative method[10] which estimates estrone, estradiol-17β and estriol separately.

In view of routine clinical applications of estrogen determinations, it seemed of interest to try to use this immunoassay in order to estimate, in urine, estrone and estradiol-17β, which represent an important fraction of estrogens in non-pregnant women.

An assay has been devised which allows a direct estimation, i.e. without any extraction or purification step. The validity of this direct radioimmunoassay was checked by comparison of the results with those obtained with the double isotope derivative technique[10] modified for urine estimation of estrone and estradiol-17β and considered as a reference method. The values obtained by the present

method were compared with those measured by the 'estrone and estradiol' fraction, isolated by chromatography and measured by both colorimetry and fluorimetry. This fraction gives an estimate of the estrogenic activity as valid as that obtained from 'total estrogens' measurements.

Such a direct assay, allowing a technician to perform 50 estimations in triplicate in 4 h, could be of great interest for the clinician, when rapid results are needed; this radioimmunoassay is simpler and faster than any other reliable method. Its main drawback, as for protein binding techniques, is a rather poor day-to-day and analysis-to-analysis reliability: however strict the definition of methodological conditions may be, the possibility exists of a sudden, unexpected fall in per cent binding, not due to the presence of estrogens, and in such techniques, there is no real means of assessing the specificity and accuracy of the measurement of a particular urine.

MATERIALS AND METHODS

Antiserum

The antiserum was kindly donated by Dr. B. V. Caldwell, Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A. It is an antiserum to estradiol-17β-succinyl-bovine serum albumin, treated with rivanol. The stock solution of antiserum is stored at $-20°C$, at a dilution of 1 : 5 in phosphate buffer. The solutions used in the experiments, at a dilution of 1 : 20,000, are kept up to one month at 4°C.

Standards

Tritiated steroids were obtained from the Radiochemical Centre, Amersham, Bucks., England: 2,4,6,7-^{3}H estrone and 2,4,6,7-^{3}H estradiol-17β with a specific activity of 100 Ci/mmol. After purification of these compounds on TLC (Benzene : Methanol, 95 : 5, v/v), a solution in ethanol is prepared, containing approximately 0·1 μCi/ml. Free steroids (estrone, estradiol-17β, estriol) were obtained from Roussel-UCLAF, Paris. Estrogen conjugates were kindly donated by Dr. A. E. Kellie, Courtauld Institute of Biochemistry, London and Dr. R. Emilozzi, Centre Hospitalier et Universitaire, Nice.

Buffer

The buffer has already been used in our plasma technique [10]. Its composition was proposed by B. V. Caldwell: 195 ml of 0·2 M NaH_2PO_4, H_2O; 305 ml of 0·2 M $NaHPO_4$, $12H_2O$; 500 ml redistilled water; 9 g NaCl; 1 g NaH_3; 2 g gelatine.

Solvents and reagents

Ethanol, from Fould Springer (SOPAR-Maisons-Alfort, France), is redistilled and treated. All other chemical compounds are from Merck.

Helix pomatia digestive juice (Industrie Biologique Française, Gennevilliers), with a titre of 130,000 Fishman units per ml of β-glucuronidase and 50,000 Roy units per ml of sulfatase is purified on disposable Pasteur pipettes (Harshaw Chemicals Ltd), plugged with glass wool and filled up to one third with Norite A. The β-glucuronidase titre is unchanged after such a purification.

METHODS

Radioactivity measurements

Radioactivity is counted in a Mark I Nuclear Chicago scintillation counter. The scintillator mixture which does not dissolve polar molecules is of standard composition: toluene, PPO, dimethyl POPOP. Tritium counting efficiency is 50 per cent in one-isotope labelling experiments and 25 per cent in dual labelling experiments; ^{35}S is counted in a channel giving a 60 per cent efficiency for ^{14}C.

Glassware washing

After prolonged soaking in a diluted Teepol solution, glassware is dipped in a chromic acid mixture for 24 h, then thoroughly rinsed with, successively, tap water, deionized distilled water, and ethanol; then, it is dried in an oven at 100°C. An alternative process is washing with DDN 150 detergent in two successive baths. Both methods seem of equivalent quality.

Urine collection and storage

Urine collected over 24 h is kept in an opaque plastic bottle, with 30 mg sodium mercurothiolate (Merseptyl).

(A) *Radioimmunoassay (Fig. 1).* Depending on the expected results, 10–20 μl of urine are pipetted into 10 ml tubes with Pedersen constriction pipettes; an equivalent volume of purified Helix pomatia digestive juice, diluted 1 : 10 with buffer for easier handling, is added and hydrolysis performed overnight at 37°C. In all tubes, the volume is adjusted with buffer to 120 μl; 200 μl of buffer containing about 10,000 d.p.m. of [^{3}H]-estradiol-17β and the antiserum are added with

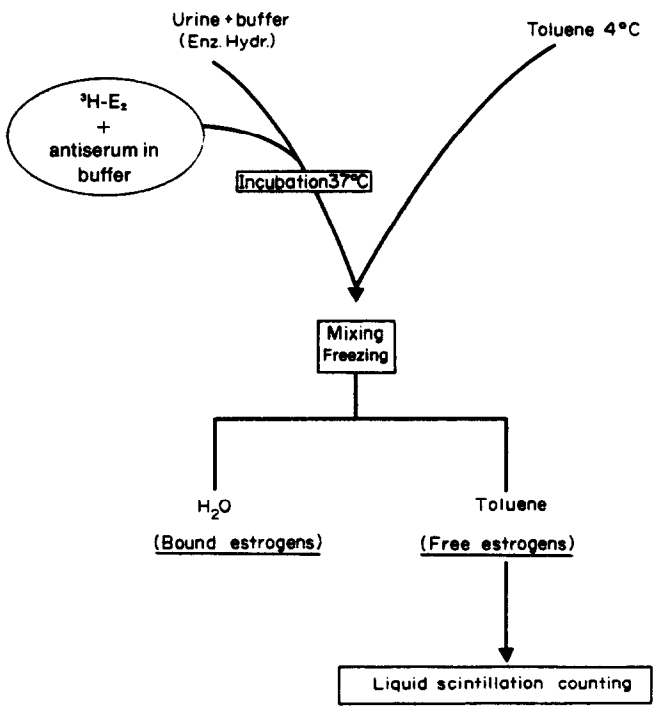

Fig. 1. Scheme of radioimmunoassay procedure.

an automatic dispenser. The final dilution of the antibody is 1:20,000. After shaking, the mixtures are incubated for 15 min at 37°C.

The separation of free and bound steroids is then achieved in a cold room (4°C) by adding 4 ml of toluene, shaking and freezing. Shaking is done on Vortex for 15 sec, then the solution is allowed to stand for 30 sec in order to get a perfect separation of the two phases. After freezing the water phase at −50°C, in a bath of ethanol and dry ice, the toluene phase is poured into a counting vial. Radioactivity of unbound molecules can then be estimated.

Standard curves (Fig. 2)

Results are read on estradiol-17β standard curves. Per cent bound radioactivity is plotted against the mass of unlabeled estrogen (log scale), ranging from 0·1 to 2 ng, each point representing the mean of triplicates. The standard tubes are prepared in the same conditions as the samples, including the introduction of an identical volume of Helix pomatia digestive juice. The results are expressed as equivalents of estradiol-17β.

The per cent bound radioactivity is estimated as follows: two tubes containing large amounts of unlabelled estradiol-17β, (so as to attempt saturation of the antiserum binding sites), allow the measurement of the maximum amount of unbound radioactivity extractable by toluene, which represents also the amount of radioactivity prone to be bound. From the difference between this value and the value of the unbound radioactivity of each sample or standard, one gets bound radioactivity values; the per cent bound value is obtained by relating this to the maximum prone to be bound.

The useful range of measurement was determined so that there was no need for urine dilution, and the dilution of the antiserum was chosen so that the standard curve would be linear and of an "efficient" slope in the considered 0·1–2 ng range.

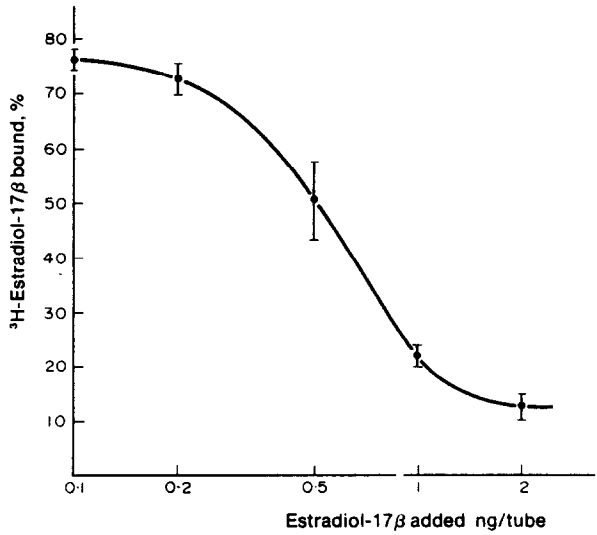

Fig. 2. Standard curve for estradiol-17β obtained with 1:20,000 antiserum dilution (mean values ± S.D., $n = 8$). No Helix pomatia digestive juice added.

Some factors influencing the assay

After addition of Helix pomatia digestive juice (10 μl), there is a significant fall in binding and a change in the slope of the standard curve (Fig. 3), both phenomena being more marked when the digestive juice is not purified. This fact led us to also perform enzymatic hydrolysis in the standard tubes, except when, on occasion, only free compounds were estimated.

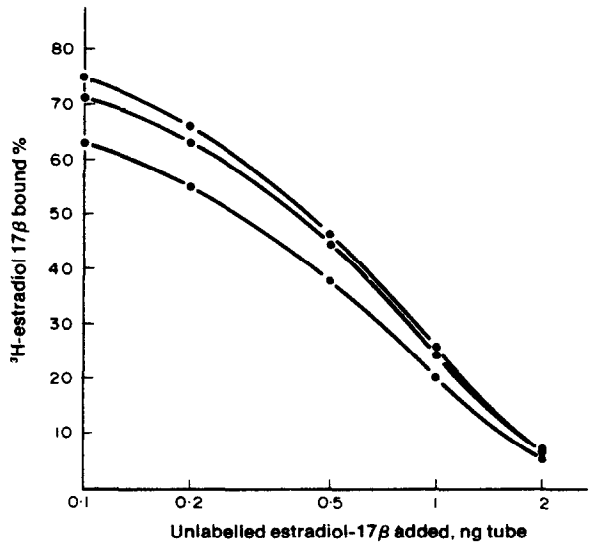

Fig. 3. Effect of Helix pomatia digestive juice (HPDJ) addition on per cent binding of estradiol-17β. Upper curve is without addition of HPDJ. Middle curve is after addition of purified HPDJ; lower curve after addition of nonpurified HPDJ.

Figure 4 shows the effect of temperature on the per cent bound radioactivity after an incubation time of 15 min. Further increase of temperature over 37°C did not improve the binding. The latter temperature was chosen for the assay. Incubation experiments from 5 to 30 min long did not show any significant difference in the level of binding.

Dilution of the antiserum leads to a change in the value of per cent binding and the shape of estradiol-17β standard curves. From examination of the latter, a suitable dilution appears to be 1:20,000; it has been used throughout the experiments (Fig. 5).

Separation of free and bound steroids is performed in a cold room with the temperature of all materials equilibrated at 4°C, since, at higher temperatures, the per cent bound radioactivity falls, even at low values of added unlabelled estradiol-17β.

(B) *Colorimetric and spectrofluorimetric methods (Fig. 6).* First, enzymatic hydrolysis is performed, either by the standard method, overnight at 37°C, or by the rapid technique at 62°C for 30 min at pH 4·5 [11].

It is followed by ether extraction: ether is washed with sodium carbonate solution, and the more polar estrogens extracted with 0·1N NaOH. Remaining estrogens are separated from neutral steroids by 1N NaOH.

After neutralisation of alkaline solution, and saturation with sodium bicarbonate, estrogens are reextracted with ether and ether extract washed with water.

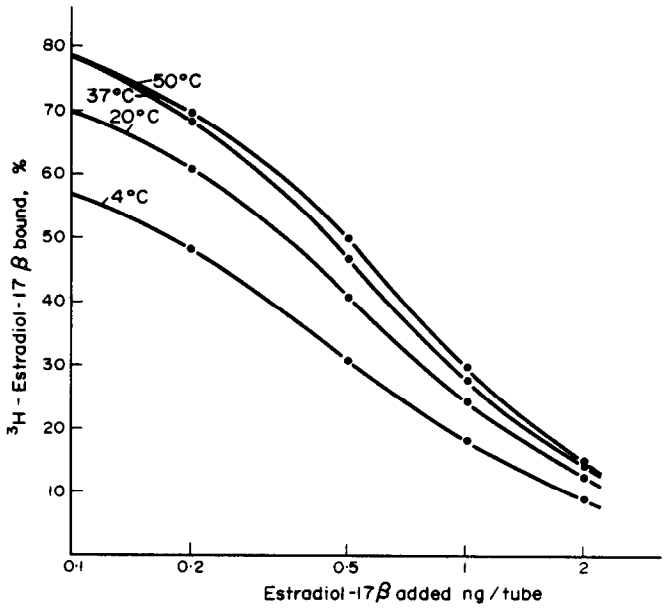

Fig. 4. Effect of incubation temperature on per cent binding of estradiol-17β (HPDJ added).

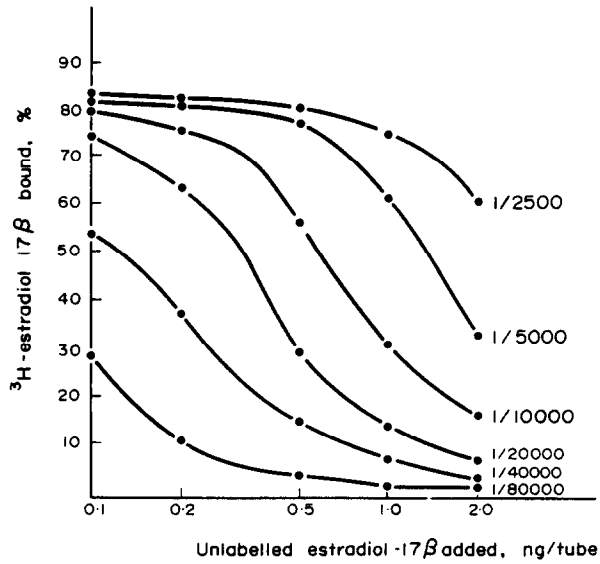

Fig. 5. Influence of dilution of antiserum on per cent binding of estradiol-17β (HPDJ added).

The extract is then chromatographed on an acidic alumina column, which permits the collection of estrone and estradiol-17β in one fraction [12].

For colorimetric measurement, this fraction undergoes a Kober reaction as described previously [13], followed by a Bradshaw type extraction [14].

For fluorimetric estimation, readings are made on a Farrand spectrofluorimeter, equipped with 2 monochromators according to the measuring physical conditions described previously [14].

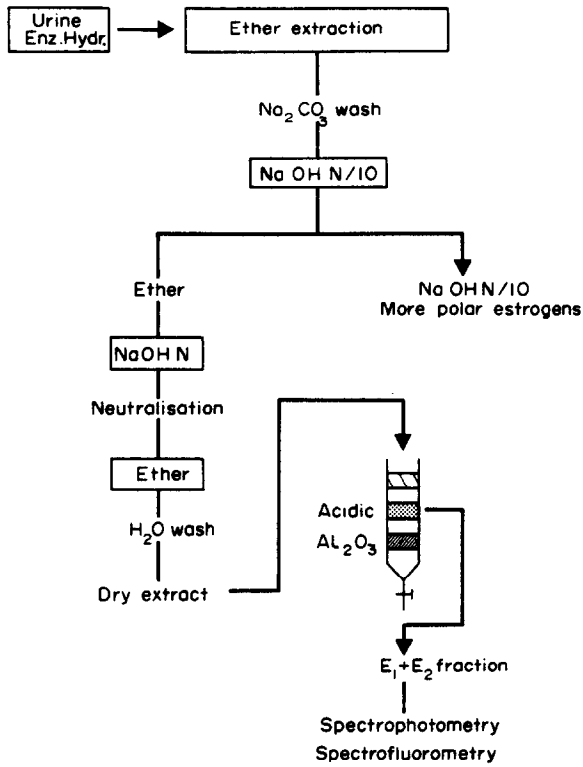

Fig. 6. Flow sheet of colorimetric and spectrofluorimetric methods.

(C) *Double isotope derivative method (Fig. 7).* The method has already been described[10] for determination of estrone, estradiol-17β and estriol in plasma. It underwent some modifications for urinary assays, and the estriol fraction is not estimated.

To 0·5 ml of urine are added tritiated indicators; then, free estrogens are extracted twice with 2 ml of ether. If conjugates are to be estimated, enzymatic hydrolysis is then performed after addition of the same amount of tritiated indicators, as in the preceding step. Such addition is valid, because the amount of radioactivity remaining in the aqueous phase after the extraction of the free compounds, is rather low and very reproducible. This amount is thus considered to be identical in all tubes, and its value is estimated by a blank tube so that, in each group of estimations, blank tubes undergo the same process as free or conjugated estrogens.

RESULTS

Specificity

Typical standard curves obtained with estradiol-17β and other compounds, at 1:20,000 antiserum dilution, are shown in Fig. 8. In such conditions, estrone and estradiol-17β are efficient ligands, but there is almost no binding for the main urine conjugates tested, which led us to perform our experiments after hydrolysis. Keeping the antiserum for 2–3 months at 4°C altered its activity but not its specificity for the tested compounds.

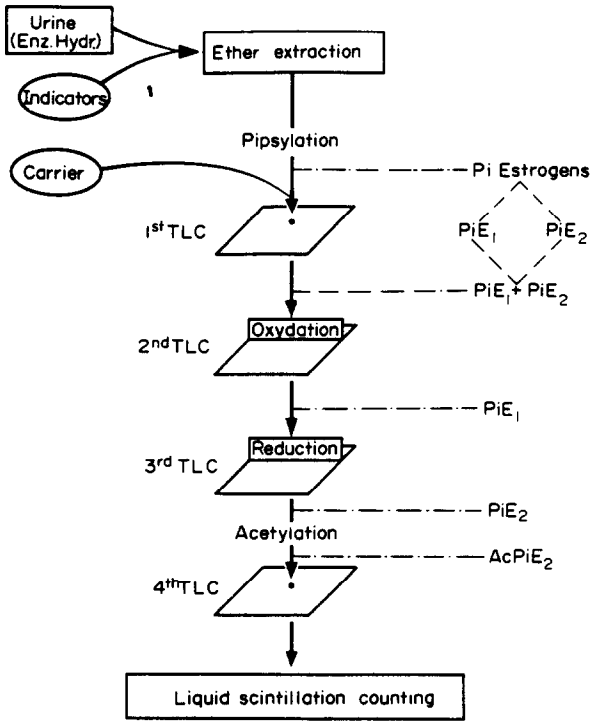

Fig. 7. Flow sheet of double isotope derivative method.

Blanks

In such a simplified direct method, the value of the water blank depends on addition of Helix pomatia digestive juice. A blank performed with 20 μl of water to which are added 10 μl of purified Helix pomatia digestive juice, is equivalent to $0{\cdot}9 \pm 0{\cdot}6$ μg per l of urine (mean value $\pm$ S.D., $n = 81$). An overestimation is found in the whole range of estrogen concentrations, which depends on the latter values (Fig. 3).

Since Helix pomatia digestive juice is the main known cause of blank value, it was decided to add it systematically to standard tubes, and have those tubes undergo the same overnight stay in 37°C oven as the samples.

Accuracy

Recovery experiments were performed for estradiol-17β. Increasing known amounts of estradiol-17β were added to a urine of a 2 year old male child. Results are shown in Fig. 9: the urinary blank value was $6{\cdot}52 \pm 0{\cdot}45$ (S.D.) μg per l. If results are corrected for blank values, the recovery is slightly superior to 100% (mean 109%, limits: 100–117%).

Precision

The reproducibility of standard curves is illustrated in Fig. 2 in which is shown the mean $\pm$ S.D. from 8 standard curves performed each in duplicate in the course of one week.

Replicate estimations were performed on urines at different concentration

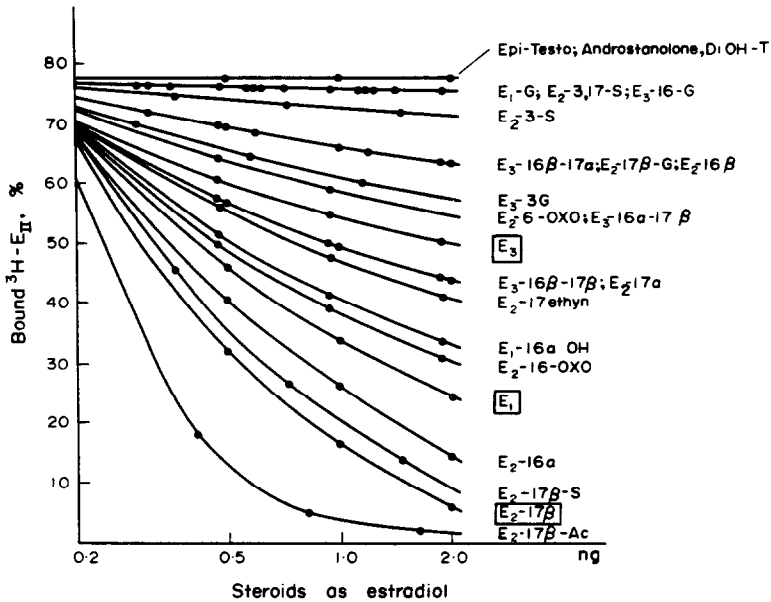

Fig. 8. Cross-reactions of various free and conjugated steroids with estradiol-17β antiserum at 1:20,000 dilution. No HPDJ added. Abbreviations for these steroids are: Testo: 17β-hydroxy-4-androsten-3-one; Androstanolone: 17β-hydroxy-5α-androstan-3-one, Di-OH-T: 4-androstene-3β,17β-diol; E_1G: 1,3,5,(10)-estratrien-17-one-3-glucuronide; $E_2$3,17-S: 1,3,5,(10)-estratriene-3,17β-disulfate; E_3-16-G: 3,17β-dihydroxy-1,3,5,(10)-estratriene-16α-glucuronide; E_2-3-S: 17β-hydroxy-1,3,5,(10)-estratriene-3-sulfate; E_3-16β-17α: 1,3,5,(10)-estratriene-3,16β,17α-triol; E_2-17β-G: 3-hydroxy-1,3,5,(10)-estratriene-17β-glucuronide; E_2-16β: 1,3,5,(10)-estratriene-3,16β-diol; E_3-3-G: 16α,17β-dihydroxy-1,3,5,(10)-estratriene-3-glucuronide, E_2-6-OXO: 3, 17β-dihydroxy-1,3,5,(10)-estratrien-6-one; E_3-16α-17α: 1,3,5,(10)-estratriene-3,16α,17α-triol; E_3: 1,3,5,(10)-estratriene-3,16α,17β-triol; E_3-16β-17β: 1,3,5,(10)-estratriene-3,16β,17β-triol; E_2-17α: 1,3,5,(10)-estratriene-3,17α-diol, E_2-17 ethyn.: 3,17β-dihydroxy-1,3,5,(10)-estratrien-17α-ethynyl; E_1-16α-OH: 3, 16α-dihydroxy-1,3,5,(10)-estratrien-17-one; E_2-16-OXO: 3, 17β-dihydroxy-1,3,5,(10)-estratrien-16-one; E_1: 3-hydroxy-1,3,5,(10)-estratrien-17-one; E_2-16α: 1,3,5,(10)-estratriene-3,16α-diol; E_2-17β-S: 3-hydroxy-1,3,5,(10)-estratriene-17β-sulfate; E_2-17β: 1,3,5,(10)-estratriene-3,17β-diol; E_2-17β-Ac: 3-hydroxy-1,3,5,(10)-estratrien-17β-acetate.

levels (6 determinations per urine). The values of the coefficient of variation are shown in Fig. 10. It decreases sharply until about 5 μg per l and then reaches a "plateau" at about 5 per cent.

A well-known phenomenon among workers in the field of radioligand assays [15] is the suddenly different behaviour between urine sample extracts and standards, the latter being depressed at all levels, with no apparent reason for such a discrepancy. Just as urinary material exerts a protective effect against estrogen destruction on alumina during column chromatography, it apparently, in some way, protects the steroid or the protein or both against structural alteration.

In order to check the quality of the standard curves, a reference sample from a urinary pool is inserted every 20 samples, in each series.

Apart from this possible overall depression of the standard curve, one is sometimes faced with an isolated tube in which the binding is not as effective as it ought to be, as is shown by replicate determination results. To cope with this

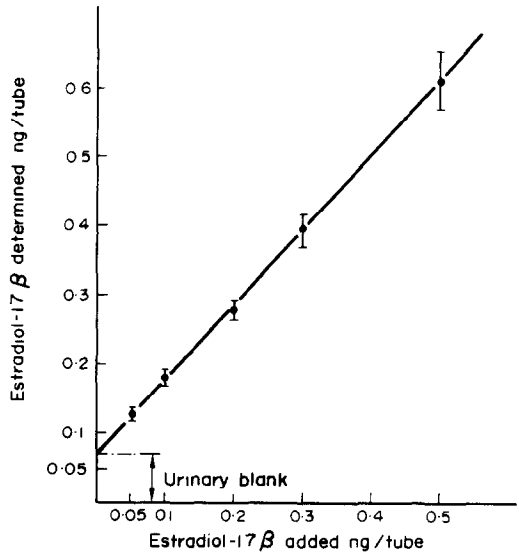

Fig. 9. Correlation between estradiol-17β added to urine of a 2 year old male child, and estradiol-17β quantified. Each point represents the mean ± S.D. of eight determinations.

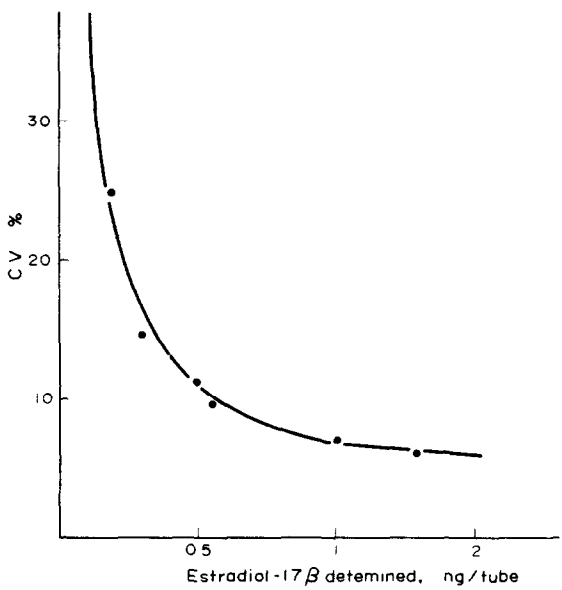

Fig. 10. Coefficient of variation (c.v.) of radioimmunoassay measurements at various concentrations of estrogens in male urine (12 determinations). No HPDJ added.

type of hazard, triplicates are done, and when the value of a sample is incidentally aberrant, then the values of the two others can still be used for the computation of the results.

Limit of detection

Taking into account the standard deviation of the water blank (0·6 μg per l), and the standard deviation of the measures at low levels, one gets a value of about 3 μg per l.

Correlations with other techniques

The correlation of radioimmunoassay with the conventional methods still used in routine work, i.e. colorimetry and fluorimetry, is not very good (Fig. 11a and 11b). This is not surprising since both are 'group' methods which do not measure exactly the same compounds, and do not evaluate the relative amount of

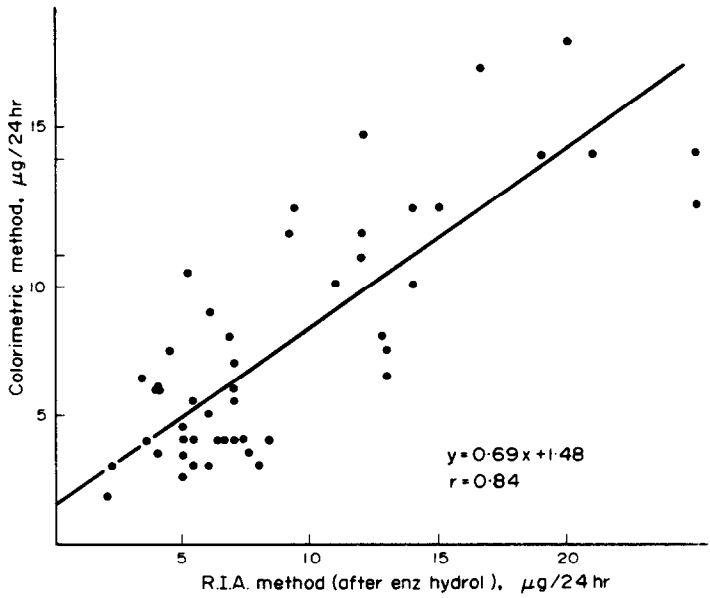

Fig. 11a.

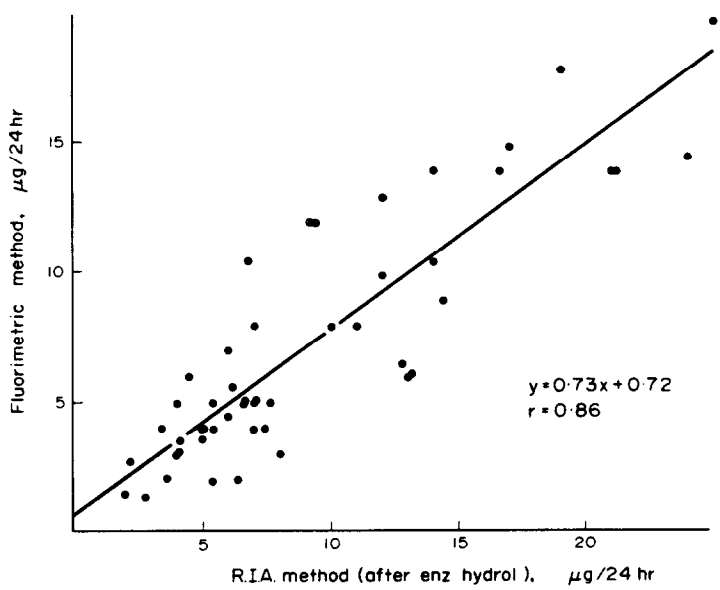

Fig. 11b.

Fig. 11. Correlation between values for urinary estrogens obtained by radioimmunoassay and values obtained by colorimetry (11a) and spectrofluorimetry (11b).

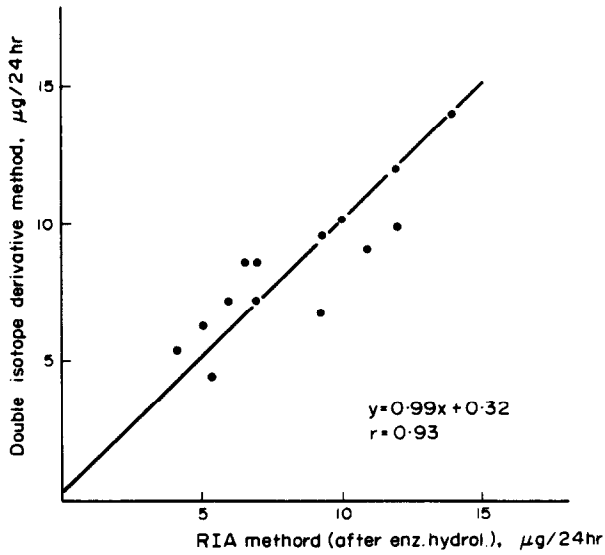

Fig. 12. Correlation between values for urinary estrogens obtained by radioimmunoassay and values obtained by double isotope derivative method.

estrogens in the same manner: in conventional methods, results are expressed as estrone equivalent, and in radioimmunoassay as estradiol-17β equivalent; furthermore, the accuracy is different in each type of method. The correlation with the double isotope derivative reference method is better (Fig. 12).

CLINICAL APPLICATION OF RADIOIMMUNOASSAY

While applying our assay to urine samples, it was decided, although the concentration of free estrogens in urine is supposed to be low, to perform in parallel the assay withour prior hydrolysis, since in the assay conditions (Fig. 8), the cross reaction of most conjugates is rather insignificant.

Figure 13 illustrates a cycle of a normal woman. The pattern shows a midcycle peak followed by an abrupt drop, then a second sustained rise. The basal body temperature shows a sharp rise leading to a 'plateau', which, in association with pregnanediol values, is a strong presumption of the existence of a luteal phase. The variation of free estrogen concentration follows the same, though less marked, pattern.

The radioimmunoassay technique was also applied to a group of normal men submitted to a dexamethasone-human chrorionic gonadotropin test (DXM-HCG test) (Fig. 14).

Figure 15 shows the results for six healthy men from a group of eleven submitted to this test. Whatever the method used, there was a definite response on day 2, which was also quite marked in blood, where estrogen estimations were made in parallel. In spite of differences in levels, it is interesting to notice that both curves always display the same general shape.

DISCUSSION

We are quite conscious that this assay may not be very satisfying from a plain scientific point of view. The mixture we are measuring is not so well defined, but

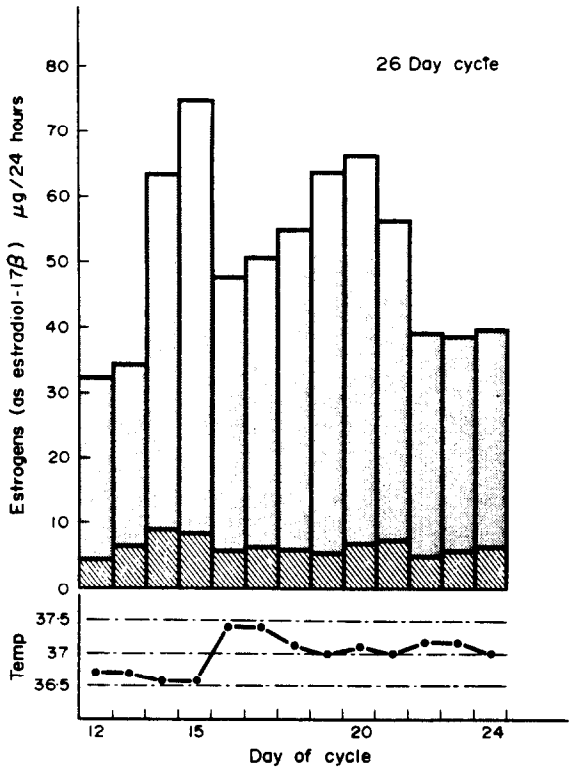

Fig. 13. "Estrone and estradiol-17β" fraction in urine during a normal menstrual cycle. Shaded areas represent concentrations measured without prior hydrolysis. Non-shaded areas represent concentrations measured after enzymatic hydrolysis.

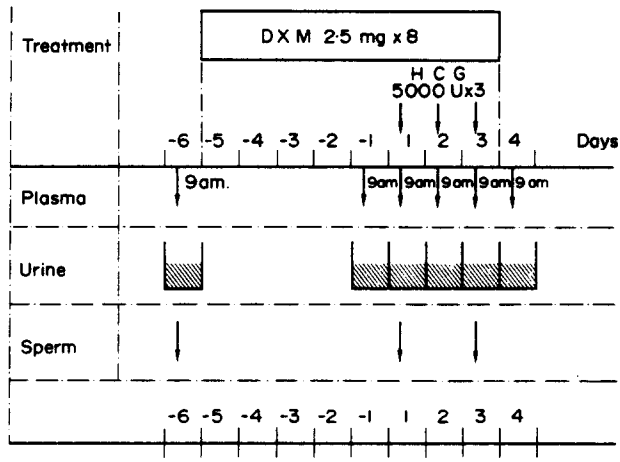

Fig. 14. Dexamethasone-Human Chorionic Gonadotropin (DXM-HCG) test design.

this has not prevented the assay from being useful in routine clinical applications.

Since Kober's work[16], quite an impressive number of techniques for estimation of estrogens in urine have been devised. Apart from pregnancy, in which estriol appears to be the predominant steroid, one must remember that it has been difficult, up to now, to assign a specific clinical meaning to any variation of the

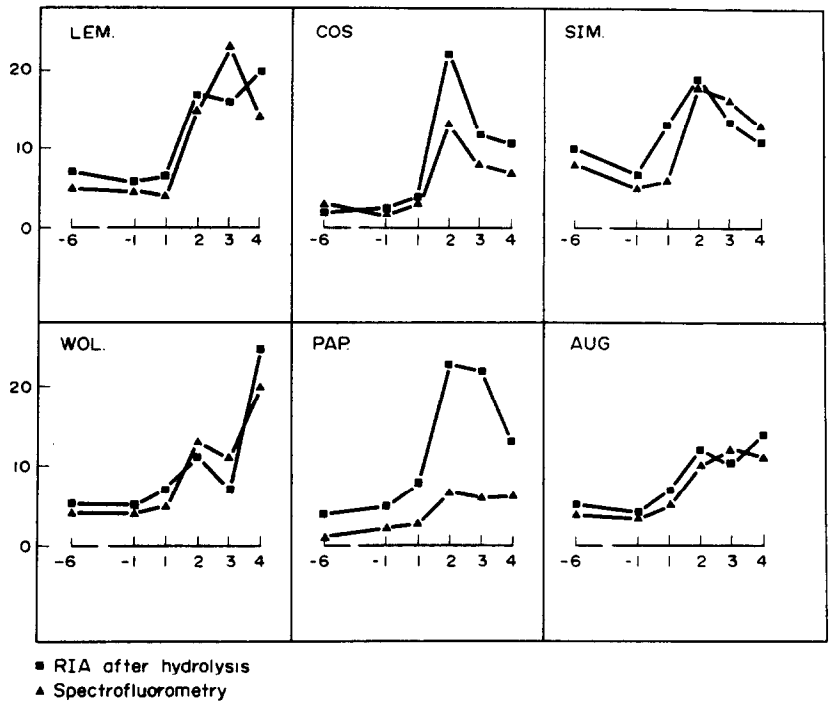

Fig. 15. Pattern of estrogen response in urine during DXM-HCG test, as measured by radioimmunoassay after hydrolysis and by spectrofluorimetry. Estrogen concentrations (μg per 24 h) are plotted on the ordinate. Day of test is plotted on the abscissa. Day 1 corresponds to the first HCG injection.

urinary concentration of a particular estrogen. In current medical practice, the assessment of estrogen production in man and woman, has been achieved in a not too cumbersome, though quite reliable way, by an estimation either of the total estrogens, or of the estrone and estradiol-17β fraction, in free and conjugated forms.

In urine, the need for a very sensitive technique is generally less imperative than in plasma. The main problem is a problem of specificity, i.e. not so much to measure a particular compound, as not to measure nonestrogenic substances. There has been, in the field of routine urine estimations outside pregnancy, a progressive evolution from very elaborate methods with many purification and separation steps, towards perhaps less refined but as useful "group" methods. A good sign of this evolution can be seen in the fact that J. B. Brown, who was pioneer and champion of drastic chromatographic separations, is himself coming back to group methods[17].

When one is actually faced with the estimation of low levels (children, menopause, strongly impaired glandular function, etc.), measurement may be made by fluorimetry[14] or gas chromatography[18–20]. The last technique is the more specific, but quite time-consuming. As for the double isotope derivative method, it will never be a routine method allowing to cope with a great number of determinations in a short time, but it is the reference method for evaluating criteria of specificity and eventually sensitivity.

With the advent of radioimmunoassay techniques, one could hope to get a

sensitive and sufficiently specific method, with the advantage of unchallenged rapidity.

However, some problems do persist in the use of such techniques. In our experience, we believe that the estimation of classical "total estrogens" still keeps its value: the greater reliability of the technique permits estimation, with less risk of large error, of a sample without any known context whether analytical (previous estimation) or clinical.

Radioimmunoassay techniques can be recommended in sequential analysis, i.e. in such treatments as induction or inhibition of ovulation, in different stimulation or stimulation suppression tests, such as dexamethasone-chorionic gonadotropin and clomiphene exploration of gonadal dysfunction. In such cases, radioimmunoassay, thanks to its simplicity and rapidity, may be the method of choice.

A further simplification of the assay would be the suppression of the hydrolysis step, through the use of a wide-spectrum antiserum. But the more aspecific the antiserum, the greater will be the hazard of also measuring non-estrogenic steroids.

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REFERENCES

1. G. E. Abraham: *J. clin. Endocr.* **29** (1969) 866.
2. N. S. Jiang, R. J. Ryan: *Proc. Mayo Clinic.* **44** (1969) 461.
3. M. Castanier and R. Scholler: *C. R. Acad. Sci. (Paris) série D* **271** (1970) 1787.
4. G. Mikhail, C. H. Wu, M. Ferin and R. L. Vande Wiele: *Steroids* **15** (1970) 333.
5. I. H. Thorneycroft, B. V. Caldwell, G. E. Abraham, S. A. Tilson and R. J. Scaramuzzi: In *Research on steroids* Vol. 4 (Edited by M. Finkelstein *et al.*) (1970) p. 205.
6. C. H. Wu, and L. E. Lundy: *Steroids* **18** (1971) 91.
7. Y. Emment, W. P. Collins and I. F. Sommerville: *Acta Endocr. (Kbh)* **67** *Suppl.* 155 (1971) 103.
8. J. Hotchkiss, L. E. Atkinson and E. Knobil: *Endocrinology* **89** (1971) 177.
9. E. Gurpide, M. E. Giebenhain, L. Tseng and W. G. Kelly: *Am. J. Obstet. Gynec.* **109** (1971) 897.
10. M. Castanier, J. Grenier and R. Scholler: *C.R. Acad. Sci. (Paris) série D* **270** (1970) 843.
11. R. Scholler, S. Métay, S. Herbin and M. F. Jayle: *Europ. J. Steroids* **1** (1966) 373.
12. R. Scholler, M. Heron and M. F. Jayle: In *Analyse des stéroides Hormonaux*, Vol. 2, (Edited by M. F. Jayle). Masson, Paris (1962) p. 397.
13. M. F. Jayle, R. Scholler, M. Heron and S. Métay: In *Analyse des stéroides Hormonaux*, Vol. 2 (Edited by M. F. Jayle). Masson, Paris (1962) p. 347.
14. R. Scholler, P. Leymarie, M. Heron and M. F. Jayle: *Acta Endocr.* **52** *Suppl.* 107 (1966).
15. B. E. P. Murphy: *Acta Endocr. (Kbh)* **64** *Suppl.* 147 (1970) 37.
16. S. Kober: *Biochem. Z.* **239** (1931) 209.
17. J. B. Brown, S. C. Macleod, C. MacNaughtan, M. A. Smith and B. Smyth: *J. Endoc.* **42** (1968) 5.
18. R. Scholler and L. Dehennin: In *Journées Hellènes de séparation immédiate et de chromatographie*, Athènes 19–24 Sept. 1965. Union des Chimistes Hellènes. Athènes.
19. H. Adlercreutz and T. Luukkainen: In *Gas phase chromatography of steroids* (Edited by K. B. Eik-Nes and E. C. Horning). Springer Verlag, Berlin (1968) p. 215.
20. H. H. Wotiz and S. C. Chattoraj: In *Gas chromatography in the analysis of steroid hormones* (Edited by H. H. Wotiz and S. J. Clark). Plenum Press, N.Y. (1966) p. 225.

DISCUSSION

De Moor: May I ask you what happens when you alter the ratio of oestrone to oestradiol in your urine, without altering the total amount of oestrogens?

Grenier: From a theoretical point of view, I can tell you that two kinds of facts

would lead to a good correlation in a urine in which estrone and estradiol are the predominent estrogens, and are present in the usual ratio of about 2:1; first, estimated values are not corrected for methodological losses, which are greater for fluorimetry than for radioimmunoassay; but, and this is the second point, this difference is in part corrected by the under-estimation of the smaller fraction (estradiol) in fluorimetry (results being expressed as equivalent of estrone, and the slope of the standard curve of the latter being slightly steeper than that of estradiol) and the underestimation of the greater fraction (estrone) in radioimmunoassay (results being then expressed as equivalents of estradiol). In any urine in which the ratio would favor estradiol, one would expect to find a difference between the results of fluorimetry and radioimmunoassay.

De Moor: So the results will change according to the ratio E_1/E_2 present in the urine—the results obtained by immunoassay will change not only according to the total amount but according to the ratio of the two components.

Grenier: That's right; according to the urine, the results will change, and this might explain the discrepancy between the results for the fifth patient in Fig. 15. But again, we are not claiming that we measure accurate amounts of specific estrogens. We are just trying to get values which can be useful for routine clinical application; and this point seems justified, at least from the results obtained during the DXM-HCG test: even when hydrolysis is not performed in radioimmunoassay, one finds a significant response, of interest to the clinician. If you have a completely impaired ovarian function, whatever the fraction you are actually measuring, you will find negligible amounts. If you have a normal ovarian function, you can have an estimate of it, even while not measuring any really specific fraction.

De Moor: The results in the fifth patient may be an artifact. According to one method it does not react, and according to another it reacts very clearly.

Grenier: As you could see in the cross-reactions figure, we have not tested all free or conjugated estrogens; it might also be that in this urine, you have got other steroids which react (I agree, we don't know what they are), and which cause the discrepancy between the two curves.