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Endogenous Oestradiol-17β Concentration in Breast Tumours Determined by Mass Fragmentography and by Radioimmunoassay: Relationship to Receptor Content

M. EDERY,* J. GOUSSARD,* L. DEHENNIN,† R. SCHOLLER,† J. REIFFSTECK† and M. A. DROSDOWSKY*

**Laboratoire de Biochimie C.H.U. Caen* 14000 *and* †*Fondation de Recherche en Hormonologie, Fresnes* 94260, *France*

Abstract—*Oestradiol-17β was determined in human breast tumours by radioimmunoassay and gas chromatography–mass spectrometry. Simultaneous determination of the oestradiol and progesterone receptor content was carried out. Oestradiol receptor positive tumours contained a greater statistically significant oestradiol concentration than receptor negative tumours.*

Our results indicate that (a) the use of gas chromatography–mass spectrometry is more valid in measuring endogenous oestradiol-17β content, (b) false negative receptor assays due to the presence of endogenous oestrogens are unlikely.

INTRODUCTION

THE VALUE of oestrogen receptor (ER) and progesterone receptor (PGR) assays as a guide for breast cancer therapy is now well established [1]. Since most receptor assays are based on the quantitative determination of the 'unoccupied' receptor, it is therefore important to know the factors influencing the receptor assay. It has been reported that ER positive tumours are more frequent among post-menopausal women whose oestrogen levels are very low [2], so that endogenous oestrogens can influence the receptor assay.

However, recent studies suggest that this situation is unlikely; it has been shown using exchange assay that endogenously bound oestradiol receptor exists in pre- and post-menopausal women [3], although the latter have a higher receptor level [4]. Furthermore, in a selected group of ER positive PGR positive post-menopausal women, Saez *et al.* demonstrated a positive correlation between the levels of ER and circulating oestrogens [5]; however, plasma oestrogens may not represent an accurate indicator of tumour status due to the existence of a high plasma–tissue gradient]6].

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Several reports have recently appeared demonstrating the presence of oestradiol-17β in human breast tumours [7–9].

Radioimmunoassay (RIA) was used in some studies, and gas chromatography–mass spectrometry (GCMS) in others. Although it has been shown that GCMS and RIA give similar results for plasma samples [10], the interpretation of RIA values for tissues remains open to criticism, because the high metabolism of the tumour tissue leads to numerous cross-reacting substances [11].

In the present work, we compare the values obtained for oestradiol-17β measured by RIA and GCMS in a large number of tumour samples. The correlation with the levels of oestradiol and progesterone receptor is also presented.

MATERIALS AND METHODS

Tissues

Samples of 78 primary breast tumour tissues were obtained fresh from biopsies or mastectomies. They were immediately chilled, trimmed free of fat, weighed and directly processed for assay, or stored in liquid nitrogen until used.

Assay of cytoplasmic receptors

For the preparation of cytosols, tissues were homogenized under continuous cooling (0–2°C) in 6 vol 10 mM Tris–HCl, 12 mM dithiothreitol and 10% glycerol buffer, pH 7.4, using short bursts with an Ultra-Turrax homogenizer. Cytosols were obtained by centrifuging the homogenates at 100,000 *g* for 1 hr in an MSE-65 ultracentrifuge. The total protein content of cytosols was determined by the method of Lowry *et al.* [12], using bovine serum albumin as the standard.

ER and PGR were determined essentially by identical procedures. The binding reaction was performed at 0–2°C by incubation of aliquots of cytosol (0.1 ml) for 18 hr with 4 nM ^{3}H-R 2858 for ER, 4 nM ^{3}H-R 5020 for PGR, either alone or in the presence of competing nonradioactive steroid (2500 nM). In order to minimize interference with nonspecific proteins, 100 nM of 5α-DHT were added to the incubation medium for ER, and 100 nM of cortisol for PGR. After incubation, bound and free steroid fractions were separated by addition of 0.12 ml of dextran-coated charcoal. After continuous shaking for 30 min at 0–2°C, the charcoal was discarded by centrifuging at 800 *g* for 10 min.

Aliquots of supernatant (0.12 ml) were added to 10 ml of scintillation fluid and radioactivity measured in a liquid scintillation spectrometer (Intertechnique SL 4000) with 61% counting efficiency for tritium. Specific binding was calculated from the difference of radioactivity bound in presence and absence of an excess of unlabelled steroid.

Extraction of steroids

Tissues were prepared as described by Millington [9] with a further purification on a Sephadex LH 20 column. The extraction yield using [2,4,6,7-^{3}H] oestradiol as tracer was 80–90%; it was independent of the amount of tissue (0.1–2 g).

Radioimmunoassay [13]

Oestradiol-17β was measured by RIA using an antiserum prepared by immunization of rabbits with oestradiol-17β-6 (*O*-carboxy) methyloxime conjugated to bovine serum albumin; the antiserum used was that referred to as L 4386 [14] and has been shown to be specific for oestradiol-17β (0.7% cross reaction of oestrone with this antiserum) [14]. Sensitivity, blank values, accuracy and inter–intra assay precisions of the assay have been previously described [13].

Derivative formation

Trimethylsilyl ethers (TMS) were used as derivatives and prepared by dissolving the extracts and standard mixtures in 100 μl of *N*, *O* bis (trimethylsilyl) trifluoroacetamide/pyridine (1:1, v/v) and heating for 1 hr at 60°C. After evaporation under a stream of N_2, the residues were dissolved in benzene and an aliquot was transferred on the solid injection needle.

Gas chromatography–mass spectrometry

The details of the procedure for GCMS have been described previously [15]. The gas chromatograph was a GIRDEL Instrument (Suresnes, France) fitted with a solid injection system and a high resolution capillary column (approximately 70,000 theoretical plates) directly coupled to a quadrupole mass spectrometer (RIBERMAG R 1010B, Rueil-Malmaison, France) The glass open tubular column (25 m × 0.3 mm i.d.) was statically coated with SE-52 stationary phase and operated at 220°C with a helium pressure of 0.8 bar.

Isotope dilution–mass fragmentography (IDMF) was performed by adding known amounts (400 pg) of deuterium-labelled internal standard [16,16,17-$^2H_3$] oestradiol-17β (E_2-d_3) to the tissue extracts before derivatization. The isotopic purity of E_2-d_3 is demonstrated by the abundance ratio $d_0/d_3 = 0.3\%$, measured on the molecular ion of oestradiol-bis (trimethylsilyl ether) at m/e 416 for E_2 and at m/e 419 for E_2-d_3.

Calculation of the amount of oestradiol-17β in the tissue extracts was made by using the peak height ratio after correction for d_0 contribution of E_2-d_3 ($d_0/d_3 = 0.3\%$) and d_3 contribution of E_2 (4%). The sensitivity, specificity, accuracy, recovery and reproducibility of the determination have been reported previously [15].

RESULTS

The endogenous concentrations of oestradiol-17β were determined in 42 human breast cancers using IDMF and RIA methods. Regression analysis of the data obtained by RIA (x) and mass fragmentography (y) gives the equation $y = 0.91x - 1.8$; the correlation coefficient is $r = 0.88$ ($P < 0.001$, Student's t-test) (Fig. 1).

However, if only the lower values (<2.8 pg/mg protein) are used for regression analysis, no correlation is found between the

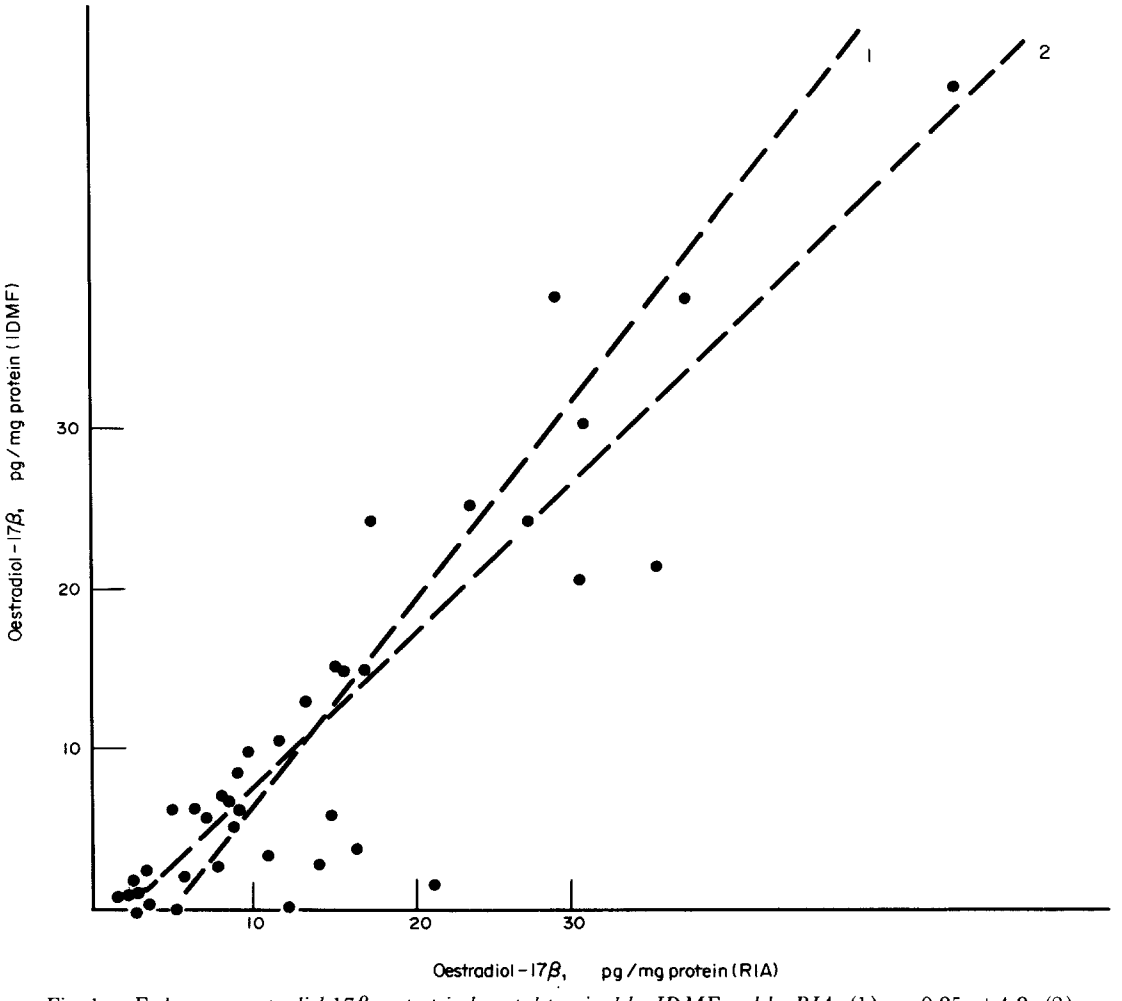

Fig. 1. *Endogenous oestradiol-17β content in breast determined by IDMF and by RIA.* (1) x=0.85y+4.8. (2) y=0.91x−1.8, r=0.88.

two methods. In fact, the RIA values are dispersed; the coefficient of variation inter assay is 36%, whereas it is 5% for mass fragmentography values. This is due to the fact that at these low levels, blank values and cross-reacting substances interfere with the RIA.

Using the IDMF methodology, oestradiol-17β was determined in 78 human breast cancers; their oestradiol and progesterone receptor contents were measured simultaneously. In ER+tumour (ER>3 fmol/mg protein), the mean value for oestradiol-17β is 15.9±7 pg/mg protein, whereas it is 5.5 ±5.6 pg/mg protein in ER− tumours (Table 1). In the ER+ group, there is no difference between PGR+ and PGR− tumours.

No correlation is found between ER content of the tumours and endogenous oestradiol-17β content both in ER+ (Fig. 2) and in ER+PGR+ (Fig. 3) tumours. However, a clear tendency to high receptor levels at high oestradiol-17β contents can be noted in the ER+PGR+ group (Table 2).

Table 1. *Mean oestradiol-17β content in breast tumours*

Receptor distribution	pg/mg protein	pg/mg protein
ER+PGR+	15.6±7 (34)*	ER+: 15.9±7 (49)
ER+PGR−	16.5±7.2 (15)	
P	NS	
ER−PGR+	7.9±7.9 (7)	ER+: 5.5±5.6 (29)
ER−PGR−	4.7±4.6 (22)	
P	NS	<0.001

*Number of cases is given in parentheses.

Although only a small number of tumours of pre-menopausal women are available, there is no evidence that pre-menopausal women had a higher oestradiol content than post-menopausal women. Furthermore, in the ER+ group, there is no significant difference in oestradiol content between pre- and post-menopausal women (Table 3), though the latter have a higher ER content (86 ±31 fmol/mg protein vs 52±12 fmol/mg protein).

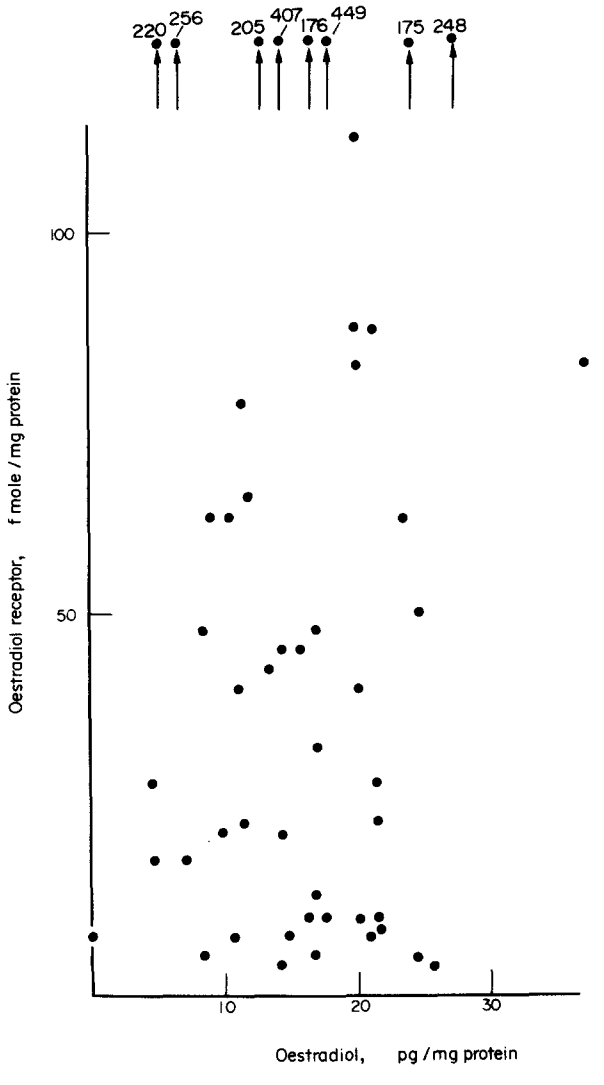

Fig. 2. ER values listed according to endogenous oestradiol-17β in ER+tumours. r = 0.06.

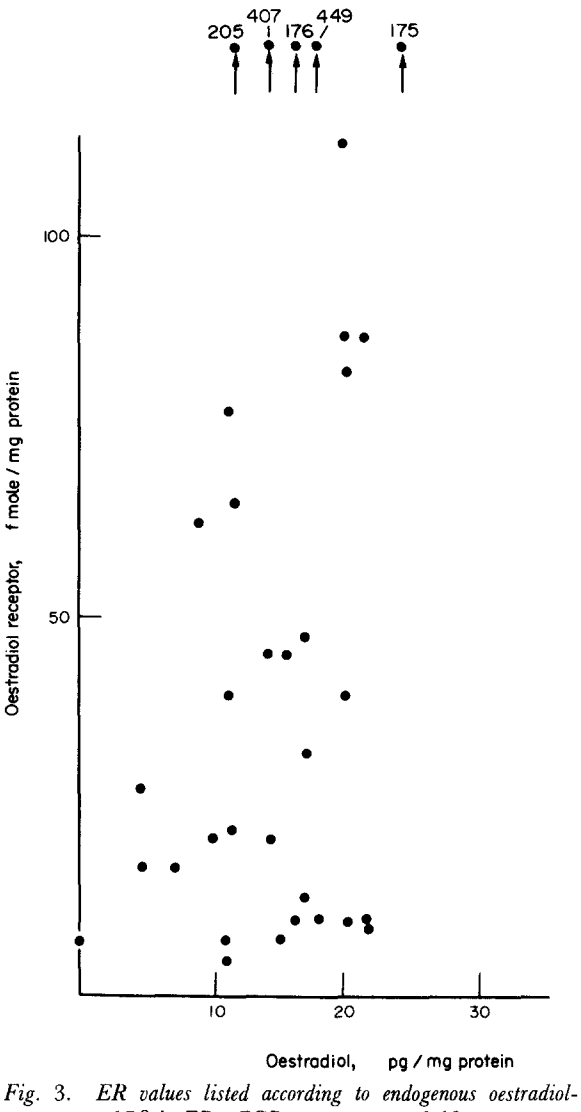

Fig. 3. ER values listed according to endogenous oestradiol-17β in ER+PGR+tumours. r = 0.19.

Table 2. ER level and oestradiol-17β content

ER level	Oestradiol-17β (pg/mg protein)
<3	5.5
3–50	13.5
50–100	18.1
≧101	19.1

Table 3. ER level and oestradiol-17β content

	pg/g tumour	Receptor status (fmol/mg protein)	pg/mg protein
Pre-menopausal (7)*	15.5±7.6	ER+: 52.6±12.2 (5)	17.1±4.1
		ER− (2)	5.6±5.7
Post-menopausal (71)	12±8.3	ER+: 86.2±31.3 (44)	15.7±7.2
		ER− (27)	5.5±5.8
P	NS		

DISCUSSION

Our data reveal that values measured by RIA and IDMF are in good agreement; however, the use of mass fragmentography seems more valid in providing an accurate methodology to measure steroid concentrations in tumour extracts, particularly when low levels of oestrogens are to be assayed.

Previous attempts using GCMS to determine oestrogens had several shortcomings, particularly the absence of correction for procedural losses. The utilization of deuterated hexamethyl [$^2H_{18}$] disilazane, monodeuterated [4-^{2}H] oestradiol-17β, dideuterated [2.4-$^2H_2$] oestradiol-17β as in-

ternal standard improved the validity of the assays. To increase assay accuracy and sensitivity, it is preferable to use three- to four-fold deuterated internal standards to avoid the natural isotope peaks surrounding the molecular ion.

We have shown that receptor-positive tumours contain a greater statistically significant oestradiol concentration than receptor-negative tumours, which is consistent with the fact that the hormone controls its own receptor synthesis. Fishman *et al.* [7] and Abul Hajj [8] presented similar results and concluded that false negative receptor assays due to the presence of endogenous oestrogens are unlikely.

The origin of such endogenous levels of oestrogen remains unclear. It is now established that mammary tumour tissue can *in vitro* synthesize oestrogens.

Nevertheless, the *in vitro* aromatase activity of the tumour may not reflect its real hormonal status; glandular secretions are preponderant in pre-menopausal women, and extraglandular aromatization of androgens (androstenedione particularly) [16] as well as adrenal secretion of oestrogens [17] are not negligible in post-menopausal women. It is also probable that in the 40–50% oestrogen receptor-positive tumours which are unresponsive to endocrine therapy, additional criteria of selection are necessary for a more precise prediction. A long term program will assess whether endogenous steroids in conjunction with receptor status can provide a better prognostic value for endocrine therapy.

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