PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 72 (2000) 259–264

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

A novel HPLC-RIA method for the simultaneous detection of estrone, estradiol and estrone sulphate levels in breast cancer tissue

Jürgen Geisler, Hildegunn Berntsen, Per Eystein Lønning*

Department of Oncology, Haukeland University Hospital, N-5021 Bergen, Norway

Received 7 July 1999; accepted 8 December 1999

Abstract

Estrogen deprivation is an effective approach for treatment of hormone sensitive breast cancer. While much is known about plasma estrogen levels with respect to castration in premenopausal women and use of aromatase inhibitors in postmenopausal women, currently there is increasing interest in intra-tumour estrogen production. However, knowledge about alterations in intra-tumour estrogen levels is limited, mainly due to methodological problems with measurements of estrogen fractions in tissue samples. Here we describe a new method for simultaneous measurement of the three main estrogen fractions, estrone (E_1), estradiol (E_2) and estrone sulphate (E_1S) in breast tumour tissue. Following incubation with [^{3}H]-labelled estrogen standards, crude fractions were separated by ether extraction. The E_1S fraction was hydrolysed with sulphatase followed by eluation on a Sephadex column. High pressure liquid chromatography (HPLC) was used to purify the individual estrogen fractions prior to RIA analysis. Estrone and E_1S were converted into E_2, and all three estrogen fractions were finally measured by the same highly sensitive and specific radioimmunoassay using estradiol-6-(*O*-carboxymethyl)-oximino-2-(2-[^{125}I]-iodo-histamine) as a ligand. Although several purification steps were used, the internal recovery values for tritiated estrogens were found to be 25–50% for E_1 and E_2 and 15–30% for E_1S. The detection limit of this method was 4.3 fmol/g tissue for E_2, 19.8 fmol/g tissue for E_1 and 11.9 fmol/g E_1S, respectively. Using tissue from locally advanced breast cancers ($n = 14$), we found median levels of E_1, E_2 and E_1S to be 283.8 fmol/g tissue (range 19.8–547.5), 554.1 fmol/g (9.5–3024.2) and 209.4 fmol/g (11.9–753.4), respectively. The method described here is a promising tool to study intra-tumour estrogen fractions in breast tissue biopsies.

1. Introduction

Estrogens are known to stimulate growth of neoplasia in hormone sensitive tissues like the breast and the endometrium [1]. Thus, for many years scientific studies have measured plasma estrogen levels to evaluate the biochemical effects of different treatment options like aromatase inhibitors. However, plasma estrogen levels do not necessarily reflect tissue estrogen concentrations. It is well-established that breast cancer cells [2] as well as connective tissue cells within tumours [3] express the aromatase enzyme, responsible for the final step in estrogen synthesis, and current evidence suggests local estrogen production to be a major pathway contributing to intra-tumour estrogen levels [4]. Moreover, while the aromatase gene in tumour cells and peripheral tissues is similar, local aromatase could be stimulated by growth factors and interleukins locally expressed in tumour tissue [5,6]. Finally, results from animal studies suggest concentration-dependent uptake of circulating estrogens to the tissue [7]. Thus, it is highly important to evaluate not only alterations in plasma but also intra-tumour estrogen levels in response to endocrine therapy.

Recent studies have revealed third generation

* Corresponding author. Tel.: +47-55-97-2010; fax: +47-55-97-2046.

E-mail address: plon@haukeland.no (P.E. Lønning).

0960-0760/00/$ - see front matter
PII: S0960-0760(00)00036-4

aromatase inhibitors to inhibit total body aromatization by 97–98% [8–10] and to suppress plasma estrogen levels by more than 90%. While several studies have evaluated estrogen concentrations in normal and malignant breast tissue of postmenopausal women [11–23], only a few studies have determined tissue estrogen levels in patients during treatment with aromatase inhibitors [24,25] due to technical problems in measuring tissue estrogens in the low range.

Here, we describe a sensitive method for the simultaneous measurement of the main estrogen fractions, estrone (E_1), estradiol (E_2) and estrone sulphate (E_1S), in malignant breast tissue. The method described here involves HPLC purification of estrogen fractions and conversion of E_1 and E_1S to E_2 followed by radioimmunoassay (RIA) analysis using an iodinated tracer.

2. Materials and methods

Radiolabelled estrogens, [2,4,6,7,-^{3}H]E_1 (101 Ci/mmol), [2,4,6,7,16,17-^{3}H]E_2 (170 Ci/mmol) and [6,7,-^{3}H]E_1S (60 Ci/mmol) for recovery determinations were obtained from DuPont NEN (Boston, MA) and estradiol-6-(*O*-carboxymethyl)-oximino-2-(2-[^{125}I]-iodo-histamine) (2000 Ci/mmol) from Amersham International (Little Chalfont, U.K.). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden), sulphatase (S-9754) from Sigma Chemical Co. (London, U.K.), and the E_2 antibody (ER 150, Sorin Biomedica S.p.A, Saluggia, Italy) from Sodiag SA (Losone, Switzerland). Methanol, acetonitrile, dichloromethane and chloroform were obtained from J.T. Baker (Deventer, The Netherlands) and ethyl acetate from Fisher Scientific (Loughborough, U.K.). All these reagents were of HPLC grade. Ethanol was obtained from Arcus AS (Oslo, Norway). Lipidex-5000 lipophilic, hydrophobic gel for liquid chromatography was obtained from Packard Instrument Corp. (Meriden, CT), sodium borohydride was from Fluka Chemie AG (Buchs, Switzerland) and sodiumdihydrogenphosphatmonohydrat from Merck (Darmstadt, Germany). The HPLC column (250 mm × 4.6 mm) and the pre-column as well as the packing material (Hypersil-5μ-ODS for both), was obtained from Hypersil Ltd. (Chesire, U.K.).

Breast cancer tissue samples were obtained from patients undergoing mastectomy at the Department of Surgery at the Haukeland University Hospital. All specimens were immediately trimmed for fat or connective tissue, dried for blood and stored in liquid nitrogen until analysis. The wet weight of every tumour sample was measured. For determination of tissue estrogen concentrations before and during therapy with an aromatase inhibitor, pre- and on-treatment samples corresponding to at least 150 mg of tissue were obtained.

To determine intra-assay coefficients of variation of this method, several grams of tissue from large breast tumours were homogenized as described below and thereafter separated into different aliquots, each corresponding to approximately 150 mg of tissue, before freezing.

The method is summarized in Fig. 1. Except where indicated, all procedures were carried out at temperatures of approximately 4°C. Tumour tissue (about 150 mg) was homogenized in 2.5 ml McIlvaine's citric acid-phosphate buffer (pH 2.75) using an Ultra Turrax T 25 (Janke and Kunkel, IKA-Labortechnik, 79219 Staufen, Germany) at 24000 rpm for 1 min. [^{3}H]Es-]Estrone, [^{3}H]E_2, and [^{3}H]E_1S (about 2000 cpm each) for internal (recovery) standards were evaporated to dryness in a vial, the homogenized sample added, and the resulting suspension was incubated for 24 h at 4°C. To enhance equilibrium, samples were treated with ultrasound (35 kHz) for 10 min at 37°C following shaking.

Unconjugated estrogens were extracted with ether (3 × 5 ml) followed by chromatography on Lipidex-5000 columns using chloroform : H_2O : methanol (9 : 1 : 2 v/v) as described by Hämäläinen [26]. The individual unconjugated estrogen fractions were subsequently separated by a high pressure liquid chromatography

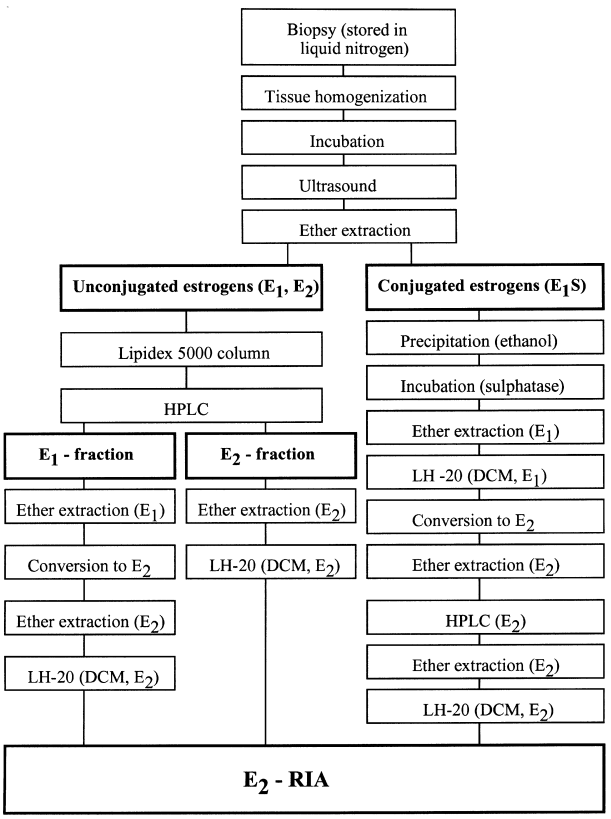

Fig. 1. Method: flow schedule of procedures.

(HPLC) system. HPLC was performed isocratically at a flow rate of 1.0 ml/min using a mixture (7 : 13 v/v) of acetonitrile and phosphate-buffer (15 mM, pH 3.5) as eluent as previously reported [27,28]. The HPLC-column was stored at room temperature. A programmable gradient mixer (Isco Model 2360, Lincoln, Nebraska) was used to de-gass the solvents used in the HPLC-system connected to a tenary HPLC pump (Model SP8800, Spectra Physics, San Jose, CA). The samples were injected using a programmable sample injector (Model 234, Gilson Medical Electronics, S.A., Villiers le Bel, France) equipped with a Rheodyne Model 7010 injector valve (Cotati, CA) and a 200-μl sample loop together with a quaternary solvent delivery system. The retention times for the different estrogen fractions were determined by injecting non-labelled estrogen standards into the HPLC system and detection by a spectrophotometric UV-detector routinely adjusted at 280 nm. The different sample fractions were collected using a Gilson Fraction Collector Model FC 204 (Gilson Medical Electronics, S.A., Villiers le Bel, France). Radiotracer labelled estrogens ([^{3}H]E_1 and [^{3}H]E_2) were used to adjust the fraction collector. The retention times for labelled and unlabelled estrogens were found to be equal.

Following HPLC-purification, the fractions containing E_1 or E_2 were pooled, respectively, and the unconjugated steroid extracted from the HPLC-buffer using ether as described above. The E_2 fraction was eluted on a LH-20 column using dichloromethane : ethyl acetate : methanol (97 : 5 : 1 by vol.) as solvent, followed by RIA as reported elsewhere [29,30]. Briefly, the fraction was reconstituted in 1 ml of methanol. Three hundred μl were obtained for recovery measurements, and aliquots of 150–200 μl used in duplicate for the RIA. Final values were corrected for recovery and for the amount of [^{3}H]-labelled hormone added as an internal standard.

The E_1-fraction was converted into E_2 using a method described previously [31]. Briefly, sodium borohydride dissolved in 0.01 M NaOH to a final concentration of 1 mg/ml in NaOH : methanol (1 : 10 by vol.) was added. The samples were incubated at 37°C for 15 min. After the evaporation of methanol, borohydride was neutralized by adding 0.5 ml of sodium-acetate buffer (0.2 M, pH 3), the E_2 fraction was extracted by ether (3 × 5 ml), purified on a LH-20 column and subjected to RIA as outlined above.

To determine the tissue concentration of E_1S, ethanol (10 ml) was added to the water fraction following extraction of the unconjugated estrogens. The sample was vortexed and centrifuged for 15 min at 1500 rpm. The ethanol fraction was removed, dried, and the residue reconstituted in 2 ml sodium-acetate buffer (0.2 M, pH 5) containing sulphatase (S-9754) to a final concentration of 2 mg/ml. Hydrolysis was performed for 48 h at 37°C followed by ether extraction of free E_1 as outlined above. Following ether extraction, the E_1 fraction was purified using LH-20 columns (dichloromethane : ethyl acetate : methanol, 97 : 5 : 1 by vol.), converted to E_2, re-purified and analyzed by RIA as given above.

Samples from a tumour-pool were used as internal standards. Briefly, we collected T_2 (2–5 cm) breast tumours and performed all tissue homogenization steps as outlined above. Finally, aliquots (n = 40–50), each corresponding to about 150 mg of tumour tissue diluted in tissue buffer were stored at −18°C until processing.

To estimate the detection limits (x_l) of the method we used repeated (n = 22) blank measurements and the equation [32]:

$$x_l = x_{bl} + k s_{bl}$$

where x_{bl} is the mean of the blank measurements, s_{bl} is the standard deviation of the blank measurements, and k is a numerical factor chosen based on the confidence level desired. We used k = 3, corresponding to a probability of 99%. All estrogen values are given as their geometric means with 95% confidence intervals of the mean.

3. Results

The dectection limits of this method are 4.3 fmol/g tissue for E_2, 19.8 fmol/g tissue for E_1 and 11.9 fmol/g tissue for E_1S, respectively. The overall recovery through all purification steps was about 25–50% for [^{3}H]E_1 and [^{3}H]E_2 and 15–30% for [^{3}H]E_1S. A typical eluation curve for radiolabelled estrogens following separation with HPLC is shown in Fig. 2.

The intra-assay coefficient of variation (CV) was determined by repeated analysis of 8–10 parallel samples obtained from a single primary breast cancer.

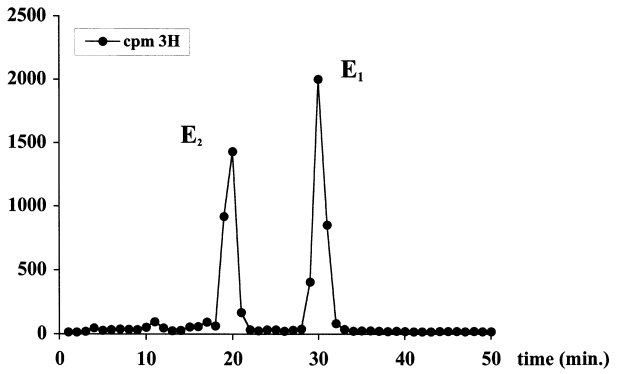

Fig. 2. Retention times for tritiated E_1 and E_2 determined in minutes after injection into the HPLC-column.

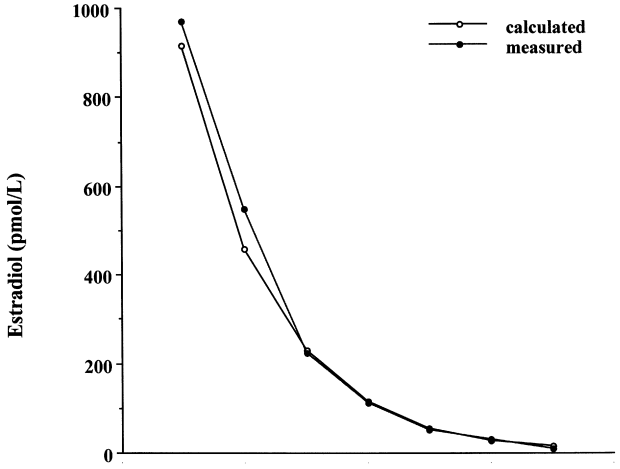

Fig. 3. Recovery of known amounts of unlabeled E_2 standards added to parallel tumour samples (compared to calculated values). Abscissa: different samples covering the range from about 15 to 1000 pmol/l.

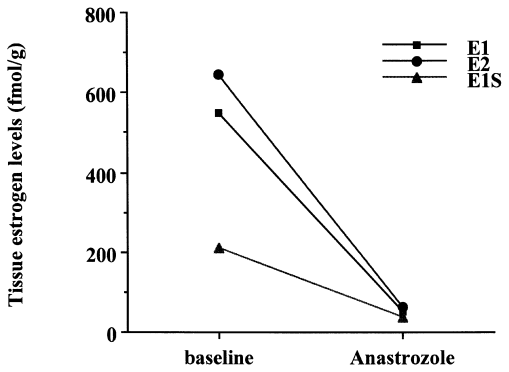

Fig. 4. Influence of neoadjuvant treatment with anastrozole (Arimidex®) on intra-tumour estrogen levels in a postmenopausal woman with advanced breast cancer ('baseline' = prior to initiation of treatment; 'Anastrozole' = after 15 weeks on anastrozole therapy).

We found a mean intra-assay CV of 6.0%, 6.7% and 6.4% for E_2, E_1 and E_1S, respectively. The inter-assay CV was determined by analysing parallel tissue samples on several occasions ($n = 4$), providing values of 19.0%, 16.0% and 20.1% for E_2, E_1 and E_1S, respectively.

For further testing of the reliability of this method, large tumour samples were homogenized as described above and separated into aliquots. Estradiol in different amounts (0–1000 pmol/l) was added, and the total concentration determined. The results of these recovery measurements for cold standard E_2 revealed a linear increase with a CV of 8.7% (Fig. 3).

Finally, we used this method to measure the concentrations of E_1, E_2 and E_1S in breast tumour samples obtained from postmenopausal women ($n = 14$) suffering from advanced breast cancer (ER and/or PGR positive). We found median levels of E_1, E_2 and E_1S to be 283.8 fmol/g tissue (range 19.8–547.5), 554.1 fmol/g (9.5–3024.2) and 209.4 fmol/g (11.9–753.4), respectively. In one of these patients, neoadjuvant treatment with anastrozole (Arimidexr®) given as 1 mg once daily for 15 weeks was found to suppress intra-tumour levels of E_1, E_2 and E_1S levels by 90.1%, 90.1% and 81.9%, respectively compared to pretreatment levels (Fig. 4).

4. Discussion

Homogenization of breast tumour tissue may be difficult due to its varying consistency, sometimes containing a high proportion of connective tissue. Following evaluation of several methods, we found the combined use of ultra-turrax and ultrasound to be the most effective procedure obtaining the highest recovery of internal [^{3}H]-labelled estrogen standards. The addition of other steps, like ultracentrifugation followed by resuspension, did not increase the percentage of recovery. This finding indicates that all major cellular compartments harbouring estrogens are reached by this homogenization method [33]. The extraction of unconjugated estrogens was performed with pure ether and, in our hands, found to be superior compared to a mixture of ethanol and acetone, although some publications have recommended the latter procedure [20].

While tumour samples of about 150 mg were used to establish this method, the method works well with samples down to a size of 100 mg.

A major problem concerning breast cancer samples is the varying amount of fat in the samples, disturbing some of the purification steps used in this method. However, as suggested in the literature [34], the application of Lipidex-5000 columns made it possible to remove excessive amounts of fat from the tumour samples with negligible influence on the hormone recovery values.

One of the main reasons to develop a new method for the detection of tissue estrogens was the variability of tissue estrogen levels published previously suggesting method difficulties [11–23]. Moreover, it is particularly difficult to determine low estrogen levels, as may be expected during treatment with the new aromatase inhibitors. Our goal was to develop a method with a high degree of sensitivity combined with high specificity. This goal was reached by the use of a highly-specific and sensitive RIA in combination with HPLC purification of the tissue samples. The combined use of a [^{125}I]-based RIA and sample purification has previously been shown [31] to reduce possible interactions by other compounds in our RIA. The HPLC eluation profile revealed a good separation of

E_1 and E_2 (Fig. 2) without any sign of interfering peaks (as determined by UV-detection).

The method described above has been shown to produce reliable results for intra-tissue estrogen levels and is applicable for the measurement of estrogen fractions in breast cancer tissue of pre- and postmenopausal women. Moreover, the low detection limits for E_1, E_2 and E_1S allow to detect changes in estrogen tissue levels in the lower range. Currently, we use the presented methodology to study alterations in tissue estrogen levels in patients receiving treatment with aromatase inhibitors.

Acknowledgements

This work was supported by grants from the Norwegian Cancer Society. We are grateful to Mr. D. Ekse for his skilful technical assistance, Dr. B. Lindtjorn (Department of Surgery, Haukeland University Hospital) for providing the tumour tissue samples, Mr. P. Skulstad (Department of Biochemical Endocrinology, Haukeland University Hospital) and Mr. J. Schneede (Department of Biochemistry, Section for Pharmacology, Haukeland University Hospital) for valuable discussion. J. Geisler is the recipient of a fellowship from the Norwegian Cancer Society. We would like to thank Zeneca Pharmaceuticals for financial support to develop this method.

References

[1] D.B. Thomas, Do hormones cause cancer, Cancer 53 (1984) 595–604.
[2] A. Lipton, S.J. Santner, R.J. Santen, H.A. Harvey, P.D. Feil, D. White-Hershey, M.J. Bartholomew, C.E. Antle, Aromatase activity in primary and metastatic human breast cancer, Cancer 59 (1987) 779–783.
[3] R.J. Santen, S.J. Santner, R.J. Pauley, L. Tait, J. Kaseta, L.M. Demers, C. Hamilton, W. Yue, J.P. Wang, Estrogen production via the aromatase enzyme in breast carcinoma: which cell type is responsible?, Journal of Steroid Biochemistry and Molecular Biology 61 (1997) 267–271.
[4] W.R. Miller, P. Mullen, J. Telford, J.M. Dixon, Clinical importance of intratumoural aromatase, Breast Cancer Research and Treatment 49 (1998) S27–S32.
[5] M.J. Reed, L. Topping, N.G. Coldham, A. Purohit, M.W. Ghilchik, V.H.T. James, Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors, Journal of Steroid Biochemistry and Molecular Biology 44 (1993) 589–596.
[6] A. Purohit, M.W. Ghilchik, L. Duncan, D.Y. Wang, A. Singh, M.M. Walker, M.J. Reed, Aromatase activity and interleukin-6 production by normal and malignant breast tissues, Journal of Clinical Endocrinology and Metabolism 80 (1995) 3052–3058.
[7] S. Masamura, S.J. Santner, P. Gimotty, J. George, R.J. Santen, Mechanism of maintenance of high breast tumour estradiol concentrations in the absence of ovarian function: role of very high affinity tissue uptake, Breast Cancer Research and Treatment 42 (1997) 215–226.
[8] J. Geisler, N. King, M. Dowsett, L. Ottestad, S. Lundgren, P. Walton, P.O. Kormeset, P.E. Lønning, Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatisation and plasma oestrogen levels in postmenopausal women with breast cancer, British Journal of Cancer 74 (1996) 1286–1291.
[9] M. Dowsett, A. Jones, S.R.D. Johnston, S. Jacobs, P. Trunet, I.E. Smith, In vivo measurement of aromatase inhibition be letrozole (CGS 20267) in post menopausal patients with breast cancer, Clinical Cancer Research 1 (1995) 1511–1515.
[10] J. Geisler, N. King, G. Anker, G. Ornati, E.D. Salle, P.E. Lønning, M. Dowsett, In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients, Clinical Cancer Research 4 (1998) 2089–2093.
[11] A.A.J.v. Landeghem, J. Poortman, M. Nabuurs, J.H.H. Thijssen, Endogenous concentration and subcellular distribution of estrogens in normal and malignant breast tissue, Cancer Research 45 (1985) 2900–2906.
[12] A. Vermeulen, J.P. Deslypere, R. Paridaens, G. Leclercq, F. Roy, J.C. Heuson, Aromatase, 17-beta-hydroxysteroid dehydrogenase and intratissue sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women, European Journal of Cancer and Clinical Oncology 22 (1986) 515–525.
[13] J. Fishman, J.S. Nisselbaum, C.J. Menendez-Botet, M.K. Schwartz, Estrone and estradiol content in human breast tumours: relationship to estradiol receptors, Journal of Steroid Biochemistry 8 (1977) 893–896.
[14] J.R. Pasqualini, G. Chetrite, C. Blacker, M.-C. Feinstein, L. Delalonde, M. Talbi, C. Maloche, Concentrations of estrone, estradiol, and estrone sulphate and evaluation of sulphatase and aromatase activities in pre- and postmenopausal breast cancer patients, Journal of Clinical Endocrinology and Metabolism 81 (1996) 1460–1464.
[15] T. Thorsen, M. Tangen, K.F. Stoa, Concentration of endogenous oestradiol as related to oestradiol receptor sites in breast tumour cytosol, European Journal of Cancer and Clinical Oncology 18 (1982) 333–337.
[16] P. Mistry, K. Griffith, P.W. Maynard, Endogenous C19-Steroids and oestradiol levels in human primary breast tumour tissues and their correlation with androgen and oestrogen receptors, Journal of Steroid Biochemistry 24 (1986) 1117–1125.
[17] M. Edery, J. Goussard, L. Dehennin, R. Scholler, J. Reiffsteck, M.A. Drosdowsky, Endogenous oestradiol-17-beta concentration in breast tumours determined by mass fragmentography and by mass fragementography and by radioimmunoassay: relationship to receptor content, European Journal of Cancer 17 (1980) 115–120.
[18] C. Recchione, E. Venturelli, A. Manzari, A. Cavalleri, A. Martinetti, G. Secreto, Testosterone, dihydrotestosterone and oestradiol levels in postmenopausal breast cancer tissues, Journal of Steroid Biochemistry and Molecular Biology 52 (1995) 541–546.
[19] D.S. Millington, Determination of hormonal steroid concentrations in biological extracts by high resolution mass fragmentography, Journal of Steroid Biochemistry 6 (1975) 239–245.
[20] J.H.H. Thijssen, A.A.J.v. Landeghem, J. Poortman, Uptake and concentration of steroid hormones in mammary tissues, Annals of the New York Academy of Sciences 464 (1986) 106–116.
[21] P.V. Maynard, B.G. Brownsey, K. Griffith, Oestradiol levels in fractions of human breast tumours, Journal of Endocrinology 77 (1978) P62–P63.

[22] R.C. Bonney, M.J. Reed, K. Davidson, P.A. Beranek, V.H.T. James, The relationship between 17-beta-hydroxysteroid dehydrogenase activity and oestrogen concentrations in human breast tumours and in normal breast tissue, Clinical Endocrinology 19 (1983) 727–739.
[23] K. Vallent, T. Feher, L. Bodrogi, Z. Ribai, Steroid-Hormon-Gehalt der Brustsubstanz bei Mammatumouren, Chirurg 53 (1982) 34–36.
[24] M.J. Reed, G.W. Aherne, M.W. Ghilchik, S. Patel, J. Chakraborty, Concentrations of oestrone and 4-hydroxyandrostenedione in malignant and normal breast tissue, International Journal of Cancer 49 (1991) 562–565.
[25] P.C.d. Jong, J.v.d. Ven, H.W.R. Nortier, I. Maitimu-Smeele, T.H. Donker, J.H.H. Thijssen, P.H.T.J. Slee, R.A. Blankenstein, Inhibition of breast cancer tissue aromatase activity and estrogen concentrations by the third-generation aromatase inhibitor vorozole, Cancer Research 57 (1997) 2109–2111.
[26] E. Hämäläinen, A micromethod for the simultaneous determination of clinically important androgens and oestrogens in plasma, Scandinavian Journal of Clinical and Laboratory Investigation 42 (1982) 493–498.
[27] P.E. Lønning, P. Skulstad, A. Sunde, T. Thorsen, Separation of urinary metabolites of radiolabelled estrogens in man by HPLC, Journal of Steroid Biochemistry 32 (1989) 91–97.
[28] S. Jacobs, P.E. Lønning, B. Haynes, L. Griggs, M. Dowsett, Measurement of aromatisation by a urine technique suitable for the evaluation of aromatase inhibitors in vivo, Journal of Enzyme Inhibition 4 (1991) 315–325.
[29] P.E. Lønning, S.I. Helle, D.C. Johannessen, H. Adlercreutz, E.A. Lien, M. Tally, D. Ekse, T. Fotsis, G.B. Anker, K. Hall, Relations between sex hormones, sex hormone binding globulin, insulin-like growth factor-I and insulin-like growth factor binding protein-1 in post-menopausal breast cancer patients, Clinical Endocrinology 42 (1995) 23–30.
[30] M. Dowsett, P.E. Goss, T.J. Powles, G. Hutchinson, A.M.H. Brodie, S.L. Jeffcoate, R.C. Coombes, Use of the aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route, Cancer Research 47 (1987) 1957–1961.
[31] P.E. Lønning, D. Ekse, A sensitive assay for measurement of plasma estrone sulphate in patients on treatment with aromatase inhibitors, Journal of Steroid Biochemistry and Molecular Biology 55 (1995) 409–412.
[32] D.D. Koch, T. Peters, Selection and evaluation of methods, in: C.A. Burtis, E.R. Ashwood (Eds.), Tietz Fundamentals of Clinical Chemistry, Saunders, Philadelphia, PA, 1996, pp. 170–181.
[33] A.A.J.v. Landeghem, J. Poortman, A. Helmond-Agema, J.H.H. Thijssen, Measurement of endogenous sub-cellular concentrations of steroids in tissue, Journal of Steroid Biochemistry 20 (1984) 639–644.
[34] V.H.T. James, J.M. McNeill, P.A. Beranek, R.C. Bonney, M.J. Reed, The role of tissue steroids in regulating aromatase and oestradiol 17-beta-hydroxysteroid dehydrogenase activities in breast and endometrial cancer, Journal of Steroid Biochemistry 25 (1986) 787–790.