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# A Sensitive Assay for Measurement of Plasma Estrone Sulphate in Patients on Treatment with Aromatase Inhibitors

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**A major obstacle to the understanding of the mechanisms of action of aromatase inhibitors in breast cancer is the observation that plasma estrogens are sustained at about 30–50% of their control levels despite 85–95% inhibition of the conversion of tracer androstenedione (A) to estrone ($E_1$). The discrepancy could be due to lack of sensitivity of current RIAs. Due to low levels of plasma estradiol ($E_2$) (mean about 20 pM) and $E_1$ (mean about 75 pM) in postmenopausal women, it is difficult to develop RIA methods with the sensitivity required to detect $>90\%$ suppression from baseline. In contrast, the plasma level of the estrogen conjugate estrone sulphate ($E_1S$) is substantially higher (mean level about 400 pM). This paper describes a new assay to measure plasma $E_1S$ in the low range aiming to detect $>95\%$ suppression of $E_1S$ from baseline values in patients treated with aromatase inhibitors. $E_1S$ was separated from unconjugated estrogens, hydrolysed and purified as unconjugated $E_1$. $E_1$ was subsequently reduced to $E_2$, purified, and measured by a highly sensitive RIA using oestradiol-6-(O-carboxymethyl) oximino-(2-[$^{125}$I]iodohistamine as ligand. The sensitivity limit of the method was 2.7 pM. Patients on treatment with the aromatase inhibitors formestane or aminoglutethimide or both drugs in concert were found to have plasma levels of $E_1S$ ranging from 3 to 274 pM with a mean suppression of 78, 86 and 95%, respectively, compared to baseline, a lower suppression than that reported in previous trials with these drugs.**

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## INTRODUCTION

Aromatase inhibition is a well-defined treatment option for breast cancer in postmenopausal women. Infusion studies applying [$^3$H]androstenedione (A) and [$^{14}$C]estrone ($E_1$) in concert have shown different aromatase inhibitors like aminoglutethimide and formestane (4-hydroxyandrostenedione) to inhibit *in vivo* aromatization by $>85\%$ [1, 2]. Despite this, plasma estrogens are sustained at 30–50% of their control levels in patients on treatment with these drugs [3–5]. An unsolved question is whether these observations may be due to non-specific cross-reactions in the RIAs or, alternatively, there may be other sources of plasma estrogens (like estrogens or estrogen like compounds in the food) in these patients.

Three plasma estrogens (estradiol, $E_2$; estrone, $E_1$, and estrone sulphate, $E_1S$) are considered of biological interest. While $E_2$ is the most potent estrogen, the plasma concentration of this estrogen is low in postmenopausal women (approx. 20 pM). Mean plasma concentrations of $E_1$ and $E_1S$ are about 75 and 400 pM, respectively [6]. While $E_1$ and $E_1S$ have little biological activity *per se*, different tissues (like breast cancer tissue) contain enzymes that may convert $E_1S$ into $E_1$ and $E_2$ [7–9]. The major production pathway of estrogens in postmenopausal women is peripheral aromatization of circulating A into $E_1$ with a minor contribution from aromatization of testosterone (T) into $E_2$ [10–12]. Plasma $E_1S$ is produced by sulphation of circulating $E_1$. The levels of $E_2$, $E_1$ and $E_1S$ are in equilibrium [13, 14]. Thus, inhibition of the peripheral aromatase should be expected to cause a similar drop in all three plasma estrogens provided the drug has no influence on other enzymes involved in estrogen disposition.

With mean plasma levels of $E_2$, $E_1$ and $E_1S$ of about 20, 75 and 400 pM, respectively, RIA methods would require a sensitivity limit of less than 1, 4 and 20 pM, respectively, to detect a 95% suppression of plasma estrogen levels in the majority of patients. Thus, a

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logical approach is to develop a sensitive assay for the relatively abundant plasma $E_1S$.

## MATERIALS AND METHODS

### *Materials*

[6,7,-$^3$H]$E_1S$ (60 Ci/mmol) and [1,2,-$^3$H]DHEA (40–60 Ci/mmol) were obtained from DuPont NEN, oestradiol-6-(O-carboxymethyl) oximino-2-(2-[$^{125}$I]iodohistamine) (2000 Ci/mmol) from Amersham International, Sephadex LH-20 from Pharmacia (Sweden), sulphatase (S-9754) from Sigma Chemical Co. (U.K.) and the antibody (ER 150, Sorin) from Sodiag SA (Losone, Switzerland). All solvents were of analytical or spectrophotometric grade and obtained from Merck AG (Darmstadt, Germany) except for ethanol which was obtained from A/S Vinmonopolet, Oslo, Norway.

### *Methods*

[$^3$H]$E_1S$ (about 400 cpm) dissolved in methanol was added to test tubes. The solvent was evaporated to dryness, plasma (2 ml) was added, and the samples were allowed to equilibrate overnight. Free (unconjugated) estrogens were extracted with ether (3 × 5 ml). The ether extracts were removed, ethanol (12 ml) was added to the water fraction, the sample vortexed, and centrifuged for 15 min at 600 ***g***. The ethanol fraction was removed, dried, and the residue reconstituted in 2 ml acetate buffer (0.2 M, pH 5) containing sulphatase (S-9754) to a final concentration of 1 mg/ml. Hydrolysis was performed for 48 h at 37°C followed by extraction of free estrogens as outlined above. $E_1$ was reconstituted in dichloromethane: ethyl acetate: methanol (97:5:1 by vol) and purified on a LH-20 column (1.6 ml). Overlap from $E_2$ and DHEA in the $E_1$ fraction was $< 2\%$. The $E_1$ fraction was evaporated to dryness and reconstituted in 0.5 ml of methanol. Sodium borohydride dissolved in 0.05 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. Thereafter, the methanol was evaporated, the borohydride neutralized by adding 0.5 ml of acetate buffer (0.2 M, pH 3.0), and $E_2$ was extracted by ether (3 × 5 ml) and purified on a LH-20 column using dichloromethane:ethyl acetate:methanol (97:5:1 by vol) as solvent. When [$^3$H]DHEA was reduced by borohydrid under similar conditions, $< 2\%$ of the radioactivity was recovered in the $E_2$ fraction after chromatography on the LH-20 column. The $E_2$ fraction was evaporated to dryness and reconstituted in 1 ml of methanol. A 500 $\mu$l aliquot was obtained for recovery measurement. From the residual 500 $\mu$l, 25 $\mu$l aliquots in duplicate were obtained and the $E_2$ concentration measured by RIA as recommended for unconjugated $E_2$ in plasma [15]. If this measurement provided $E_2$ values outside the optimal part of the standard curve, repeated measurements were performed using aliquots of 50, 100 or 150 $\mu$l as appropriate. Final values were corrected for the amount of [$^3$H]$E_1S$ added. Cross-reactivity of the antibody against $E_1$ and estriol ($E_3$) was $< 3\%$, while cross-reactivity against A, T and DHEA was $< 0.1‰$.

## RESULTS

The detection and sensitivity limit of the assay [6] were 2.6 and 2.7 pM, respectively. Considering the recovery through the purification steps, about 50% of the $E_1S$ was recovered as $E_1$ after hydrolysis and extraction and 35–40% as $E_2$ after purification prior to RIA.

The intra-assay coefficient of variation (CV) was determined by repeated analysis of $E_1S$ in a plasma pool obtained from postmenopausal women (concentration of 350 pM). Six to nine parallel samples were determined on 5 different occasions providing a mean CV of 5.9%. Inter-assay CV was 14.9% at the same concentration. In addition, we determined intra-assay CV in two plasma pools made from postmenopausal breast cancer patients on treatment with the aromatase inhibitor aminoglutethimide. At plasma concentrations of 13 and 59 pM, CVs of 9.2% ($n = 10$) and 7.0% ($n = 23$), respectively, were found.

Plasma $E_1S$ was determined in 9 patients before and during treatment with formestane, aminoglutethimide, or the two drugs in concert. The results are shown in Table 1. The lowest value of $E_1S$ recorded was 3 pM. However, there was a substantial variation between individual patients with on-treatment values up to 274 pM. Treatment with formestane, aminoglutethimide and the two drugs given in concert suppressed plasma levels of $E_1S$ by mean values of 78, 86 and 95%, respectively.

## DISCUSSION

Recent studies have reported that different aromatase inhibitors, like aminoglutethimide, formestane and fadrozole, given as single drugs or in concert, inhibit *in vivo* aromatization by about 85–90% [1, 2, 16]. Despite this, plasma estrogens are suppressed only by mean values of 50–70% [3–5, 17].

The discrepancy between the percentage aromatase inhibition and plasma estrogen suppression in patients on treatment with aromatase inhibitors could be due to alternative estrogen sources. One study suggested direct production of estrogens from DHEA not involving androstenedione as an intermediate [18]. Another possibility could be estrogens obtained from food sources. Alternatively, the results may be caused by lack of sensitivity or non-specific interactions in the RIAs. Because no reference method (HPLC, GC–MS) for plasma estrogen measurement at a low range is available, this problem can only be addressed indirectly by comparing results obtained with different RIAs.

*Table 1. Examples of plasma levels of $E_1S$ before and during treatment with aminoglutethimide and formestane. Values expressed as pM (and % of control values)*

| Before treatment | Time on treatment | | | | |
|---|---|---|---|---|---|
| | 1–2 months | 3–4 months | 5–8 months | 9–12 months | 12 + months |
| 275 | 40 (15%)* | 14 (5%)* | — | — | — |
| 336 | 137 (41%)* | — | — | 133 (40%)* | 38 (11%)‡ |
| 288 | — | 41 (14%)* | 9 (3%)‡ | — | — |
| 1177 | — | 274 (23%)* | — | 54 (5%)‡ | — |
| 319 | 16 (5%)† | — | — | — | — |
| 632 | 134 (21%)† | — | — | — | — |
| 795 | 3 (1%)† | — | — | — | — |
| 261 | — | 104 (40%)† | — | — | — |
| 577 | — | 16 (3%)‡ | 20 (4%)† | — | 19 (3%)‡ |

*On treatment with formestane.
†On treatment with aminoglutethimide.
‡On treatment with formestane and aminoglutethimide in concert.

The aim of this study was to develop a RIA suitable for measurement of plasma $E_1S$ at the low range expected during treatment with aromatase inhibitors. Converting $E_1S$ into $E_2$ may be beneficial for two reasons; first, it offers the possibility of using a $^{125}I$-labelled $E_2$ with a specific activity 20-fold higher than $[^3H]E_1$. This may improve the sensitivity of the assay. Recent studies on novel aromatase inhibitors like letrozole [19] have revealed plasma estrogen levels below the sensitivity limits of the methods in a substantial number of patients when the group revealed a mean estrogen suppression of 70–80%. Second, this method involves two different steps of purification isolating the compound as $E_1$ and $E_2$, respectively. These steps of purification eliminates DHEAS from the sample by $> 99.9\%$. While the cross-reactivity of the antibody against DHEA is $< 0.1‰$, plasma levels of DHEAS are in the range of 1–3 $\mu M$ [6], and even a small cross-reactivity might interact with the result when measuring plasma levels of $E_1S$ down to a level of 3 pM.

Previous studies found most patients to have plasma $E_1S$ levels $> 100$ pM during chronic treatment with aromatase inhibitors like aminoglutethimide and formestane [5, 14]. When these two drugs were given in concert, patients achieved plasma $E_1S$ levels ranging from 53 to 119 pM [20]. In the group of 9 patients investigated in this study, 5 patients had plasma $E_1S$ levels of $\leqslant 20$ pM on at least one occasion during treatment. Treatment with formestane, aminoglutethimide and the two drugs in concert suppressed plasma levels of $E_1S$ by 78, 86 and 95%, respectively. Tracer studies have revealed formestane and aminoglutethimide, administered on the same drug schedules, to inhibit *in vivo* aromatization by 84.8 and 90.6%, respectively [1, 2], and the two drugs in concert to inhibit aromatization by a mean value of 94.8% [21]. It is also noteworthy that aminoglutethimide enhances the metabolism of plasma $E_1S$ [22]. While this effect contributes to the fall in plasma $E_1S$ during treatment with aminoglutethimide, the result obtained in the small number of patients presented here suggests a percentage of plasma $E_1S$ suppression approaching the percentage of aromatase inhibition observed with the same drugs.

Using this new assay, we measured plasma $E_1S$ levels as low as 3 pM. However, there was a substantial variation between individual patients in as much as some patients had sustained levels of plasma $E_1S$ as high as 274 pM. Thus, while the mean percentage of $E_1S$ suppression is better than that reported in previous studies using different RIAs, our results from a small number of patients suggest a larger variation in individual $E_1S$ suppression than that recorded previously. Thus, an interesting target for future studies would be to compare the degree of $E_1S$ suppression among responders and non-responders to different aromatase inhibitors using this method.

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## REFERENCES

1. MacNeill F. A., Jones A. L., Jacobs S., Lønning P. E., Powles T. J. and Dowsett M.: The influence of aminoglutethimide and its analogue rogletimide on peripheral aromatisation in breast cancer. *Br. J. Cancer* **66**, (1992) 692–697.
2. Jones A. L., MacNeill F., Jacobs S., Lønning P. E., Dowsett M. and Powles T. J.: The influence of intramuscular 4-hydroxyandrostenedione on peripheral aromatisation in breast cancer patients *Eur. J. Cancer* **28A** (1992) 1712–1716.
3. Santen R. J., Worgul T. J., Lipton A., Harvey H., Boucher A., Samojlik E. and Wells S. A.: Aminoglutethimide as treatment of postmenopausal women with advanced breast carcinoma. *A. Int. Med.* **96** (1982) 94–101.
4. Dowsett M., Cunningham D. C., Stein R. C., Evans S., Dehennin L., Hedley A. and Coombes R. C.: Dose-related endocrine effects and pharmacokinetics of oral and intramuscular 4-hydroxyandrostenedione in postmenopausal breast cancer patients. *Cancer Res.* **49** (1989) 1306–1312.
5. Johannessen D. C., Adlercreutz H., Fotsis T. and Lønning P. E.: Plasma and urinary oestrogens in breast cancer patients on treatment with 4-hydroxyandrostenedione. *Br. J. Cancer* **68** (1993) 393–398.

6. Lønning P. E., Helle S. I., Johannessen D. C., Adlercreutz H., Lien E. A., Tally M., Ekse D., Fotsis T., Anker G. B. and Hall K.: Relations between sex hormones, sex hormone binding globulin, insulin-like growth factor-I and insulin-like growth factor binding protein-1 in postmenopausal breast cancer patients. *Clin. Endocr.* 42 (1995) 23–30.
7. Santner S. J., Feil P. D. and Santen R. J.: *In situ* estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. *J. Clin. Endocr. Metab.* 56 (1984) 29–33.
8. Pasqualini J. R., Gelly C., Nguyen B.-L. and Vella C.: Importance of estrogen sulfates in breast cancer. *J. Steroid Biochem.* 34 (1989) 155–163.
9. Beranek P. A., Folkerd E. J., Ghilchik M.W. and James V. H. T.: 17-beta-hydroxysteroid dehydrogenase and aromatase activity in breast fat from women with benign and malignant breast tumours. *Clin. Endocr.* 20 (1984) 205–212.
10. Grodin J. M., Siiteri P. K. and MacDonald P. C.: Source of estrogen production in postmenopausal women. *J. Clin. Endocr. Metab.* 36 (1973) 207–214.
11. Kirschner M. A., Cohen F. B. and Ryan C.: Androgen-estrogen production rates in postmenopausal women with breast cancer. *Cancer Res.* 38 (1978) 4029–4035.
12. Hausknecht R. U. and Gusberg S. B.: Estrogen metabolism in patients at high risk for endometrial carcinoma. II The role of androstenedione as an estrogen precursor in postmenopausal women with breast cancer. *Am. J. Obstet. Gynec.* 116 (1973) 981–984.
13. Ruder H. J., Loriaux L. and Lipsett M. B.: Estrone-sulfate: production rate and metabolism in man. *J. Clin. Invest.* 51 (1972) 1020–1033.
14. Lønning P. E., Johannessen D. C. and Thorsen T.: Alterations in the production rate and the metabolism of oestrone and oestrone sulfate in breast cancer patients treated with aminoglutethimide. *Br. J. Cancer* 60 (1989) 107–111.
15. Dowsett M., Goss P. E., Powles T. J., Hutchinson G., Brodie A. M. H., Jeffcoate S. L. and Coombes R. C.: Use of aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route. *Cancer Res.* 47 (1987) 1957–1961.
16. Lønning P. E., Jacobs S., Jones A., Haynes B., Powles T. J. and Dowsett M.: The influence of CGS 16949A on peripheral aromatisation in breast cancer patients. *Br. J. Cancer* 63 (1991) 789–793.
17. Dowsett M., Stein R. C., Mehta A. and Coombes R. C.: Potency and selectivity of the non-steroidal aromatase inhibitor CGS 16949A in postmenopausal breast cancer patients. *Clin. Endocr.* 32 (1990) 623–634.
18. Longcope C., Bourget C. and Flood C.: The production and aromatization of dehydroepiandrosterone in post-menopausal women. *Maturitas* 4 (1982) 325–332.
19. Iveson T. J., Smith I. E., Ahern J., Smithers D. A., Trunet P. E. and Dowsett M.: Phase I study of the oral aromatase inhibitor CGS 20267 in postmenopausal patients with advanced breast cancer. *Cancer Res.* 53 (1993) 266–270.
20. Lønning P. E., Dowsett M., Jones A., Ekse D., Jacobs S., MacNeil F., Johannessen D. C. and Powles T. J.: Influence of aminoglutethimide on plasma oestrogen levels in breast cancer patients on 4-hydroxy-androstenedione treatment. *Breast Cancer Res. Treat.* 23 (1992) 57–62.
21. MacNeill F. A., Jacobs S., Lønning P. E., Powles T. J. and Dowsett M.: Combined treatment with 4-hydroxyandrostenedione and aminoglutethimide: effects on aromatase inhibition and oestrogen suppression. *Br. J. Cancer* 69 (1994) 1171–1175.
22. Lønning P. E., Kvinnsland S., Thorsen T. and Ueland P. M.: Alterations in the metabolism of oestrogens during treatment with aminoglutethimide in breast cancer patients. Preliminary findings. *Clin. Pharmacokinet.* 13 (1987) 393–406.