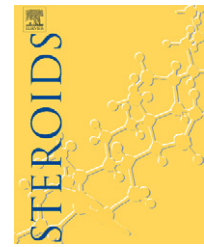


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Superiority of gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol for monitoring of aromatase inhibitor therapy

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ABSTRACT

Currently available radioimmunoassay methods for estradiol in serum lack sufficient sensitivity and precision to monitor estradiol levels in patients placed on third generation aromatase inhibitors. We recently validated a gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol and determined estrogen levels in normal post-menopausal women and in women with breast cancer before and during administration of aromatase inhibitors. Validation of the GC/MS/MS assay in human plasma and human serum included determination of assay sensitivity (<0.63 pg/ml), precision (all CVs less than 17.8%), recovery (98–103%), and linearity of recovery ($R=0.998$). Levels of estradiol were lower when assayed by GC/MS/MS compared to RIA under all conditions (7.26 ± 4.82 pg/ml versus $11.9 + 12.0$ pg/ml in normal post-menopausal women; 5.88 ± 3.43 pg/ml versus 13.8 ± 7.5 pg/ml in breast cancer patients prior to treatment; and <0.63 pg/ml versus 5.8 ± 4.1 pg/ml during aromatase inhibitor therapy). Fifty-five women treated either with atamestane/toremiphene or letrozole/placebo were monitored for estradiol levels at 4, 8 and 12 weeks of therapy. The mean levels of estradiol during aromatase inhibitor therapy was 5.8 ± 4.1 pg/ml as measured by RIA and <0.63 pg/ml by GC/MS/MS. The degree of suppression with the aromatase inhibitors as detected by RIA was 58% versus >89% by GC/MS. These results suggest that most RIA methods detect cross-reacting estrogen metabolites and yield higher measured levels than GC/MS/MS. Several pharmacological and clinical considerations suggest that GC/MS/MS should become the preferred method for monitoring aromatase inhibitor therapy.

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1. Introduction

Post-menopausal women with hormone dependent breast cancer commonly receive aromatase inhibitors to block

the conversion of androgens to estrogens [1]. Assessment of the degree of suppression of estrogen levels requires highly sensitive and precise methods for measuring estradiol. Radioimmunoassays (RIA) for plasma estradiol in current use

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lack the sensitivity and specificity to measure the low levels of estradiol found in post-menopausal women [2,3]. As a reflection of this problem, the median estradiol levels reported in normal post-menopausal women are highly variable and range from 5 to 27 pg/ml [3]. Mean levels of serum estradiol measured during suppression with aromatase inhibitors range from 2 to 10 pg/ml [4–28]. Ultra-sensitive recombinant yeast and HeLa cell bioassays have been developed in an attempt to overcome this problem [2,3,29–31]. With these bioassays, the median basal level of estradiol prior to treatment is much lower, (i.e. 2 pg/ml) and suppresses to <0.1 pg/ml with third generation aromatase inhibitors. However, these bioassay methods are not widely available, are difficult to employ, require careful attention to blank material contained in serum, and are not entirely specific for estradiol.

Determining the degree of suppression of estrogen in women receiving aromatase inhibitors requires assays which are an order of magnitude more sensitive than the assays used for measuring normal basal levels in post-menopausal women. During the development of the aromatase inhibitors, the lack of highly sensitive serum assays limited the ability of investigators to precisely quantitate the degree of suppression of estradiol. To circumvent this problem, isotopic kinetic methods were utilized and demonstrated that the third generation aromatase inhibitors suppress estradiol by 90–99% [9,13–15,17,22,32]. However, these isotopic methods require administration of relatively large amounts of radioactivity, are quite difficult to perform, and cannot be routinely utilized in women receiving aromatase inhibitors.

A key question often asked is whether it is necessary to measure estradiol accurately in women receiving aromatase inhibitors. One reason given is to document that levels are indeed adequately suppressed in individual patients. Lack of compliance, unrecognized drug/drug interactions, and genetically altered pharmacokinetics could influence the degree of suppression [33,34]. The rapidity of bone loss induced by aromatase inhibitors might also correlate with either basal or suppressed levels of estradiol. With currently available radioimmunoassay methods, lack of adequate suppression can go unrecognized and assessment of the degree of suppression can be quite imprecise.

For the reasons described, our long standing objective has been to develop highly sensitive and specific estradiol assays for use in breast cancer patients or in women at risk of developing breast cancer [2,3]. In our hands, the yeast ultra-sensitive recombinant DNA bioassay performed well and demonstrated suppression of estradiol to levels of 0.07 pg/ml with the aromatase inhibitor, letrozole [2]. However, this assay is technically quite difficult to perform and is not widely available. The HeLa cell recombinant estradiol bioassay also proved feasible but is essentially limited to research investigations [3].

During the evaluation of the HeLa cell assay, we validated our results with a gas chromatography/ tandem mass spectrometry assay (GC/MS/MS) and concluded that GC/MS/MS may be the preferred method for measuring low levels of estradiol in serum [3]. Accordingly, we performed the current study which utilized GC/MS/MS to determine the degree of suppression of estradiol in women receiving two different aromatase inhibitors. We fully validated this assay and compared results found with our standard RIA method. The GC/MS/MS

assay detected lower basal values and demonstrated a greater degree of suppression of estradiol with aromatase inhibitors than with the radioimmunoassay method. These results suggest that GC/MS/MS should receive widespread application as the preferred method to measure serum estrogens in women receiving aromatase inhibitors.

2. Methods

2.1. GC/tandem mass spectrometry assay

Deuterated estradiol was added as an internal standard to 1 ml of plasma or serum samples to enable quantitation of recovery and calculation of mass in the detected peak [3]. One milliliter of plasma or serum was extracted on BondElut Certify solid phase cartridges and eluted with ethyl acetate. The samples underwent three derivatization procedures to improve selectivity and specificity, including reaction with pentafluorobenzoylchloride (PFB); *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxyamine hydrochloride and MSTFA. The derivatized samples were then separated by gas chromatography on a DB-17 fused silica capillary column with detection by tandem mass spectrometry using negative ion chemical ionization. Development and validation of the assay was performed at Taylor Technology, Inc., Princeton, NJ.

2.2. RIA

The radioimmunoassay utilized has been previously described [3,25] and involves extraction, celite column chromatography, use of a high affinity antibody against estradiol, and 125 I-labeled estradiol tracer. This assay can distinguish 5 pg/ml of estradiol from blank with 95% confidence limits. The coefficients of variation for this RIA are 4.3% within assay and 11.4% between assay. This assay correlates with GC/MS/MS with an R value of 0.98.

2.3. Patients

Three groups of patients provided blood samples for this study. The first group included 30 normal post-menopausal volunteers. The second group included 29 post-menopausal women with breast cancer who were randomized to receive toremifene 60 mg and atamestane 500 mg daily by mouth. The third group included 26 post-menopausal women with breast cancer randomized to receive letrozole 2.5 mg daily and placebo. Written informed consent was obtained from all patients and the protocol was approved by each participating institution. The women receiving aromatase inhibitors were entered into a trial sponsored by Intarcia Pharmaceuticals, Emeryville, CA and involved centers in St. Petersburg and Moscow, Russia. The normal post-menopausal women also signed an informed consent as part of participation in a study at the University of Virginia to validate the HeLa cell bioassay.

2.4. Protocol

Women with breast cancer provided a blood sample for determination of estradiol prior to treatment with an aromatase

inhibitor and again on weeks 4, 8 and 12 while on therapy. Bloods were collected, allowed to clot, centrifuged, and the serum frozen for measurement of estradiol by GC/MS/MS and by radioimmunoassay.

2.5. Plasma sample availability

The first priority for assay measurement by study design was radioimmunoassay and sufficient volume of plasma was available for all patients. For the GC/MS/MS assay, 1 ml of sample (the amount needed for maximal assay sensitivity) was available for 23 women in the toremifene/atamestane group and 19 in the letrozole/placebo group. The results from these samples were used to calculate baseline means and standard deviations. During therapy, 136 serum samples of at least 1 ml volume were available from 40 patients on either arm of the study receiving the aromatase inhibitor on at least week 4, 8, or 12. These were used to determine the level of suppression of estradiol during administration of the aromatase inhibitor. To ensure that exclusion of the samples with insufficient plasma volumes did not skew the results, we assumed that undetectable values were at the lower limit of detection and recalculated data. This recalculation changed mean baseline values by only 0.1 pg/ml (data not shown).

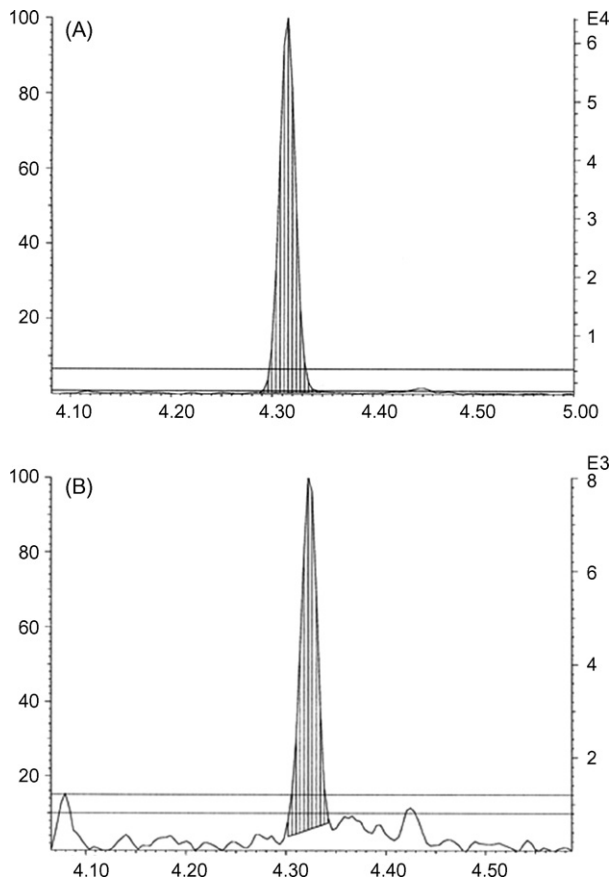


Fig. 1 – Top panel. Estradiol standard peak as it appeared in the gas chromatography fractions. Bottom panel. Estradiol peak in a sample containing 2.1 pg/ml of estradiol as it appeared in the gas chromatography fractions.

3. Results

3.1. GC/MS/MS validation

The percent recovery of estradiol added to charcoal stripped human plasma and serum averaged 79.3% with a CV of 7.12% and 87.6% with a CV of 7.01% respectively. Examples of the GC peaks for a sample spiked with estradiol and a patient's sample with a level of estradiol of 2.1 pg/ml are shown in Fig. 1 A and B. The level of sensitivity of the GC/MS/MS assay when extracting 1 ml of plasma or serum is 0.63 pg/ml. Within assay coefficients of variation (CV) were determined on three separate days for samples with low (1.9 pg/ml), medium (25 pg/ml), and high (61 pg/ml) estradiol level. Between assay CVs were calculated from these results. As shown in Table 1, no within assay CV exceeded 8.5% and no between assay CV was greater than 7.9%. Recoveries of added estradiol in samples with varying levels of estradiol were 98.9%, 98.8% and 99.8%, respectively and the linearity of recovery of added estradiol was $R=0.997$. For human serum samples, a single within day evaluation was performed. The % CVs for serum samples with low (2.1 pg/ml), medium (26 pg/ml) and high (62 pg/ml) estradiol levels were 17.8%, 3.13% and 3.27% and the recoveries were 94%, 100.4% and 103%, respectively. The linearity of response was $R=0.998$.

3.2. Comparison of basal estradiol levels measured by GC/MS/MS and by RIA

3.2.1. Normal post-menopausal women

Basal levels of estradiol in normal post-menopausal women were 7.3 ± 4.8 pg/ml (mean \pm S.D.) with GC/MS/MS and 12 ± 12 pg/ml (mean \pm S.D.) with RIA [3]. The correlation coefficient for values measured by both assays was $R=0.98$. Correlation between body weight and plasma estradiol provided an independent biologic means to validate the GC/Mass

Table 1 – Within and between assay precision of measurement of plasma estradiol (E_2) with GC/MS/MS assay

	Low E_2	Medium E_2	High E_2
Day 1			
Mean	1.80	24.8	61.1
CV	6.44	1.69	2.88
N	6	6	6
Day 2			
Mean	1.92	24.4	60.7
CV	6.51	1.75	2.44
N	6	6	6
Day 3			
Mean	1.61	23.8	60.6
CV	8.51	5.63	3.17
N	5	8	6
Between assay			
Mean	1.84	24.3	60.8
CV	7.88	3.72	2.7
N	17	18	16

spec assay. The R value for this correlation was 0.67 for GC/MS and 0.53 for RIA.

3.2.2. Post-menopausal women with breast cancer

For patients randomized to atamestane plus toremiphene, the mean pretreatment level of estradiol with GC/MS was 5.3 ± 3.0 pg/ml (mean \pm S.D.). For those in the letrozole/placebo group, the mean was 6.6 ± 3.9 pg/ml. The mean values in the two breast cancer groups did not differ significantly which allowed pooling of results with means of 5.9 ± 3.4 pg/ml with GC/MS/MS. When measured by RIA, the mean value of the pooled samples was 14 ± 7.5 pg/ml.

3.2.3. Levels of estradiol during administration of an aromatase inhibitor

Both RIA and GC/MS were used to assess estradiol levels in the group of women receiving the aromatase inhibitor atamestane plus toremiphene and in the group given letrozole plus placebo. One-hundred and thirty-six serum samples of at least 1 ml volume were available from 40 patients (on either arm of the study) who had received the aromatase inhibitor on at least week 4, 8, or 12. The estradiol level in all 136 of these samples was found to be <0.63 pg/ml. In 35 samples from 15 women, volumes were less than 1 ml but estradiol was also undetectable in each sample. If calculations are limited to those in whom sufficient sample (i.e. 1 ml) was available, there was no statistically significant differences between the two groups (i.e. 100% undetectable) and the mean values were <0.63 pg/ml. When the RIA results from both groups were pooled, the mean levels were 5.8 ± 4.1 pg/ml. Comparison of the degree of suppression of estradiol as detected by either assay (Fig. 2) demonstrated 58% suppression with the RIA method and $>89\%$ by the GC/MS method.

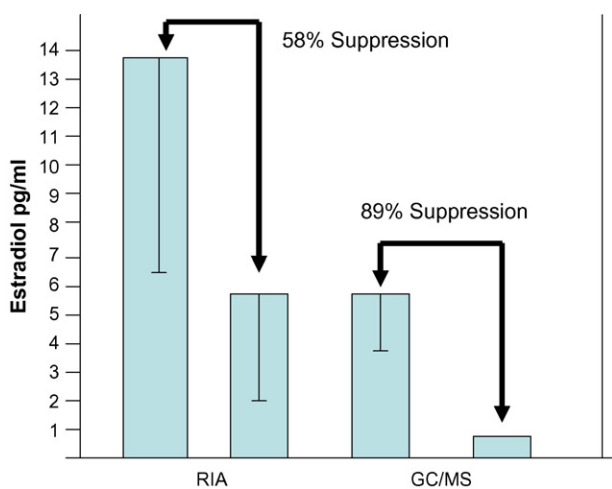


Fig. 2 – Mean levels of estradiol in the 55 women measured prior to and during therapy. The error bar represents one standard deviation. The percentage suppression is calculated from the means. In patients receiving letrozole, 100% of samples were below 0.625 pg/ml. In those receiving the toremiphene/atamestane combination, 84.6% were below the limit of detection.

4. Discussion

Methodologic and clinical data demonstrated the superior sensitivity and precision of GC/MS/MS as a measuring tool for serum estradiol when compared to a widely used and validated RIA method for assessing estradiol suppression during aromatase inhibitor therapy. When measured by GC/MS/MS, the levels of estradiol in women receiving aromatase inhibitors were found to be much lower than previously reported by RIA [4–28]. Three major factors serve to enhance the performance of GC/MS/MS: use of derivatization, a deuterated internal standard in each sample, and gas chromatography that produces a highly focused peak. In addition, the specificity derived from quantitation of the ion fragment mass peak with tandem mass spectrometry markedly improves performance. The GC/MS/MS assay utilized in this study performed exceedingly well with respect to precision, recovery of added estradiol, and sensitivity of detection.

Prior results indicate that most RIA methods lack sufficient sensitivity to accurately measure the low basal estradiol levels found in post-menopausal women and assessment of the suppressed levels observed with aromatase inhibitor therapy is even more difficult to measure with RIA [2,3]. Because of the direct nature of its detection method, GC/MS/MS is usually considered as the “gold standard method” to which other methods are compared. It is not widely appreciated that the measurement of estradiol in post-menopausal women with RIA methods is problematic. As a reflection of the lack of its specificity, RIA uniformly detects a higher level of estradiol than does GC/MS/MS. In the current study, the basal estradiol levels were substantially higher with RIA than by GC/MS/MS measurements (e.g. 12 versus 7.3 pg/ml in normal women and 14 versus 5.9 pg/ml in breast cancer patients). During the administration of an aromatase inhibitor, the mean levels obtained were 5.8 pg/ml with RIA and <0.63 pg/ml with GC/MS/MS. A comprehensive study comparing GC/MS/MS to 8 different RIA methods found higher estradiol levels with 7/8 different RIA methods (Lee, J, Cummings S, personal communication). The 8th assay detected similar levels but showed only a weak correlation between body weight and estradiol ($R=0.22$) compared to $R=0.67$ for the GC/MS/MS assay. These findings suggest that most RIAs likely detect steroidal compounds other than estradiol which cross react with the antibody used to measure estradiol and are not removed by a chromatography step. These problems become exaggerated when direct assays omitting the chromatography step are used.

During the development of aromatase inhibitors, the degree of aromatase inhibition as assessed by the isotopic kinetic techniques always differed from that determined by RIA. For example, all of the third generation aromatase inhibitors inhibited this enzyme by 90–99% as measured by the isotopic kinetic method [9,13–15,17,22,32]. By RIA, the level of suppression ranged from 50% to a maximum of 80% [4–28]. In several reports, the level of estradiol was still within the range of detection of the assay. Based upon the results obtained with the GC/MS/MS assay, one can conclude with some certainty that the RIA methods detect “blank” material in plasma or serum which probably represents estrogen metabolites.

An alternative approach for assessment estrogen levels in post-menopausal women, receiving aromatase inhibitors is the measurement of estrone sulfate [35,23]. The levels of this steroid conjugate in post-menopausal women are 50–100-fold higher than unconjugated estradiol. Assay sensitivity is therefore not a significant problem when using this assay. However, estrone sulfate is not in itself an active estrogen and must be de-conjugated to become biologically active. Therefore, this method represents an indirect means of assessing estrogen production but does provide information regarding the circulating estrogen reserve in these patients.

Cost, technical expertise and availability have until now limited the use of GC/MS/MS as a routine diagnostic tool in most steroid testing laboratories. GC/MS/MS assays are now available in most commercial reference laboratories and in academic reference laboratories such as the Mayo Clinic. While cost still exceeds that for RIA, the volume/price ratio should come down as utilization increases and lower costs for GC/MS/MS methods.

Several problems with this study should be noted. Adequate sample (i.e. 1 ml) was not available to allow an assessment of full GC/MS/MS assay sensitivity in 8 of 55 women at baseline. The initial study design planned to measure estradiol by RIA only and priority was given to this assay. For those with insufficient sample, we assumed that the basal levels were at the limit of detection of the GC/MS/MS assay based upon assay volume. This approach did not appear to skew the results since deletion of these 8 samples from the calculations changed the mean levels of estradiol from 5.9 to 5.8 pg/ml. A full 1 ml of sample was also lacking in 36 specimens from 15 women receiving the aromatase inhibitor and estradiol was undetectable in each of these samples. It should be noted that estradiol was also undetectable in 136 of the 136 samples in which a 1 ml volume was available. Accordingly, it did not seem appropriate to extrapolate values for the 36 samples with insufficient volumes. Another problem with the study was that one group of women received atamestane plus toremiphen whereas the other group was given letrozole plus placebo. It is unlikely that the toremiphen influenced the estradiol levels and it would appear that atamestane suppressed estradiol to the same extent as letrozole.

Our results demonstrated the superiority of GC/MS/MS over RIA but did not address the clinical necessity for such precise measurements in post-menopausal women. We suggest that such measurements could be practically useful in several instances. One area of utility might involve the pharmacologic considerations accompanying aromatase inhibitor use. A highly sensitive assay for estradiol may be able to identify patients who are either non-compliant, have drug-drug interactions, or possess genetic pharmacokinetic factors that would explain their lack of complete suppression [34]. Women with the greatest suppression of estradiol might also experience an increased risk of bony fractures than those with higher levels [1].

Another area where the measurement of baseline serum estradiol levels in post-menopausal women can be of use is to predict who will develop breast cancer over the subsequent 10 years. Standard RIAs of estradiol demonstrate that women in the top quintile of estradiol levels have a 2.5-fold greater chance of developing breast cancer than those in the lowest

quintile [36,37]. However, more sensitive and specific assays for serum estradiol may provide even greater predictive power. Prospective studies will now be necessary to compare the ability of baseline estradiol levels to predict breast cancer using GC/MS/MS versus RIA.

GC/MS/MS may also be useful in the assessment of who will respond to a SERM with reduction of breast cancer risk. A study involving the MORE trial demonstrated that women with low estradiol levels did not experience a reduction of breast cancer risk whereas those in the higher range did [38]. However, to precisely quantitate the very lowest levels of estradiol, the current GC/MS/MS method will have to be modified by extracting 4 ml of plasma to lower the sensitivity of the method to 0.125 pg/ml with subsequent validation of this alteration.

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