Published in final edited form as:

Horm Mol Biol Clin Investig. 2015 September ; 23(3): 79–84. doi:10.1515/hmbci-2015-0026.

Reproducibility of an assay to measure serum progesterone metabolites that may be related to breast cancer risk using liquid chromatography-tandem mass spectrometry

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Abstract

Recent data suggest a novel role of progesterone in breast cancer etiology involving the progesterone metabolites 3 α -dihydroprogesterone (3 α HP), 5 α -dihydroprogesterone (5 α P) and 20 α -dihydroprogesterone (20 α HP). Accurate and precise measures of progesterone metabolites are needed for etiologic studies of hormonally-related cancers. We have developed a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure five hormones including progesterone, its precursor pregnenolone, and three progesterone metabolites: 5 α P, 3 α HP, and 20 α HP. Hormone levels were measured in serum from 20 healthy volunteers (7 men, 5 premenopausal women, 8 postmenopausal women). Two blinded, randomized aliquots per individual were assayed in each of four batches. Coefficients of variation (CV) and intraclass correlation coefficients (ICC) were calculated from the individual components of variance. The overall laboratory CVs were <3% and ICCs were uniformly high (>98%) for all hormones measured across sex/menopausal status groups. Our LC-MS/MS assay of progesterone metabolites demonstrated excellent sensitivity, laboratory reproducibility, and interindividual variation, suggesting that this serum assay is suitable for use in epidemiologic research. The high sensitivity of the assay and thus the ability to quantify concentrations among postmenopausal women and men, further supports that this novel assay is suitable for studies of serum progesterone metabolite concentrations and risk of breast cancer or other hormonally-related cancer.

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Disclosure statement: The authors have nothing to disclose.

Keywords

reproducibility; serum progesterone assay; liquid chromatography tandem mass spectrometry; breast cancer risk

A central role for both endogenous androgens and estrogens in the etiology of female cancers is well established by laboratory and epidemiologic evidence [1, 2], while for men, evidence for a role of these hormones in prostate, testicular and breast cancer carcinogenesis is suggestive [3–5]. However, a role for progesterone in cancer development is not clear. Some menopausal hormone formulations of estrogen plus progestogen increase breast [6, 7], and ovarian cancer risk [8], but decrease endometrial cancer risk [9, 10]. Studies of menopausal hormone use and colon cancer also suggest a decreased risk with progestin exposure [11–13].

In women, laboratory evidence implicates progesterone in the development of breast and possibly other tumors, but efforts to understand the mechanism of its action and to assess risk in populations independent of other hormones in the sex steroid pathway are challenging [14, 15]. Progesterone's actions are tissue-specific and occur in concert with other hormones; for instance, in women, along with estrogen, it is a proliferative agent in the normal breast, but not in the endometrium. Less is known of the role of progesterone in men, where it is produced by the adrenals and testes, and where levels are comparable to those in postmenopausal women.

Recent data suggest a novel role of progesterone in breast cancer etiology involving the progesterone metabolites 3 α -dihydroprogesterone (3 α HP), 5 α -dihydroprogesterone (5 α P) and 20 α -dihydroprogesterone (20 α HP). Laboratory studies by Wiebe and colleagues suggest that the relative concentration of cancer-inhibiting (3 α HP) to cancer-promoting (5 α P) metabolites may promote breast carcinogenesis [16]. Experimentally, 20 α HP also demonstrates anti-mitogenic properties [17] and may selectively control aromatase activity [18] in breast cancer cell lines. Although the ovary is the primary source of progesterone in premenopausal women, after menopause, much smaller amounts are produced in adipose tissue [19, 20], and recent studies suggest local production of 3 α HP and 5 α P metabolites may also occur in the breast [16].

Few epidemiologic studies have investigated levels of endogenous progesterone in relation to cancer risk [21, 22], in part, because current assays are not able to detect the low levels observed in serum of postmenopausal women and men and require large sample volumes. In a study of postmenopausal breast cancer [21], the lower limit of detection of the progesterone assay was 3 ng/dL and almost 30% of samples had undetectable levels. Furthermore, the available commercial kits measure only progesterone, and do not measure additional metabolites which may play a role in the carcinogenic process.

In order to evaluate the role of progesterone and the metabolites 3 α HP, 5 α P, and 20 α HP in large epidemiologic studies, accurate, sensitive, precise, and relatively simple laboratory methods that require small volumes of biological samples are needed. We have developed a stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS)

method that requires 0.4 mL of serum to measure pregnenolone (progesterone precursor), progesterone, 5 α P, 3 α HP, and 20 α HP (Figure 1). The purpose of this report is to evaluate laboratory variability for our assay based on a formal reproducibility study in which blinded, randomized serum samples from men, premenopausal women, and postmenopausal women were assayed over several weeks and to assess the interindividual variability of metabolite levels by sex and menopausal status.

Hormone levels were measured in serum provided by 20 healthy volunteers that were recruited from the Research Donor Program administered by the Frederick National Laboratory for Cancer Research's Occupational Health Services in Frederick, MD under an approved protocol. Informed consent was obtained from each research donor. Donors included 7 men ages 35 to 63 years, 5 premenopausal women ages 27 to 36 years, and 8 naturally postmenopausal women ages 54 to 65 years. Donors were identified only by code, and the specimens were further de-identified using laboratory accession numbers. Donors reporting use of hormone supplements, oral contraceptives, or menopausal hormones within 3 months of blood draw were ineligible. Because the purpose of this study was to assess the performance of the assay method, we did not select donors based on other characteristics suspected of modifying pregnenolone/progesterone profiles, such as age, race, anthropometry, physical activity, diet, or stress.

Details of our analytic method for measuring serum pregnenolone, progesterone and three progesterone metabolites, including sample preparation, assay conditions, and internal standards are described in the online supplement. The steps included extraction with hexane, derivatization with hydroxylamine, followed by analysis with stable isotope dilution high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-MS/MS). Briefly, 20 μ L of internal standard solution and 0.5 mL of freshly prepared phosphate buffered normal saline solution were added to 0.4 mL human serum. Following inverse extraction with 5 mL hexane for 30 minutes, the sample was transferred to a clean glass tube and evaporated to dryness at 60°C under a stream of nitrogen gas. The dried sample was then derivatized using 100 μ L of 0.1 M hydroxylamine solution with 5% acetic acid and heated at 60°C for 30 minutes to form the oxime derivatives of hormone metabolites and their stable isotope labeled internal standards. After derivatization, all samples were analyzed by HPLC-MS/MS selected reaction monitoring (SRM) in positive ion mode using a Thermo TSQ™ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) coupled with a Prominence UFLC system (Shimadzu Scientific Instruments, San Jose CA) and quantified using Xcaliber™ Quan Browser (Thermo Scientific). During oxime derivatization of ketosteroids such as those analyzed in this study, two geometric stereoisomers can be formed from a single ketosteroid. To avoid undesirable twin peaks for a single steroid, the chromatography condition described in this manuscript ensures each metabolite is eluted as a single oxime derivatized ketosteroid peak.

All calibration and quality control samples in double charcoal stripped human serum were prepared following the same procedures as that of unknown serum samples. Analyses of calibration and quality control samples for the hormones measured performed well, suggesting excellent assay accuracy, precision, and quantitative ranges. The lower limit of quantitation is 0.5 ng/dL and limit of detection is 0.1 ng/dL.

Interindividual differences in the distribution of pregnenolone/progesterone measurements across sex/menopausal status groups were compared using box plots. Hormone values were log-transformed to stabilize variances. To quantify reproducibility of the pregnenolone/progesterone assays, estimates of the variability among subjects, assay variability among batches for a given subject, and assay variability associated with different aliquots in the same batch for the same subject were obtained using a nested component of variance analysis. Using the variance estimates we computed the overall coefficients of variation (CV) and intraclass correlation coefficient (ICC) for each sex/menopausal status group.

The serum levels of the hormones measured by HPLC-MS/MS in 20 research donor samples are shown in Table 1. Of the hormones measured, 3 α HP was consistently the lowest concentration metabolite in circulation by rank across all sex/menopausal status groups. The ranking of the other hormones measured varied across groups.

Figure 2 graphically summarizes the design of the reproducibility study for each of the five hormones measured by sex/menopausal status. The hormone concentrations are plotted on the y-axis by batch on the x-axis. The blinded duplicates for each hormone measured are shown for men and postmenopausal women, respectively. A similar plot for premenopausal women is provided in Supplemental Figure 1. The overall laboratory CVs were <3% and the ICCs were exceptionally high (>99.0%) for all hormones measured across sex/menopausal status group (Table 2).

For 3 α HP, we observed values below the lower limit of quantitation for the assay (0.50 ng/dL) for one man (3 α HP = 0.45 ng/dL) and two postmenopausal women (3 α HP = 0.28 and 0.47 ng/dL), however, all values were above the lower limit of detection (0.10 ng/dL). All other hormone levels measured were above the lower limit of quantitation for the assay, even among postmenopausal women, where levels are presumed to be the lowest.

Our novel, LC-MS/MS assay of pregnenolone, progesterone, and three progesterone metabolites demonstrated excellent laboratory reproducibility. The uniformly high ICCs for the metabolites measured suggest that interindividual variability was much greater than laboratory variation, even in postmenopausal women and men, where circulating levels are low. We also observed substantial interindividual variability of circulating levels of the three progesterone metabolites measured as well as progesterone precursor pregnenolone across sex/menopausal status groups.

Given that our results are based on a small number of participants (n=20), it is possible that the variance estimates may not adequately represent the underlying population values and provide imprecise estimates of the relevant statistics. However, the excellent reproducibility, sensitivity, and high ICCs across all sex/menopausal status groups, especially among postmenopausal women and men where concentrations are lowest, are notable. The small number of subjects in each sex/menopausal status group also limits our ability to explore hormone profiles with clinical, demographic, and anthropometric characteristics and argues for the need to evaluate these associations in large population-based studies. Finally, the use of single blood draw from research donors does not address intra-individual variation in progesterone metabolite profiles.

In conclusion, our findings of excellent laboratory reproducibility and interindividual variation in progesterone metabolites suggest that this serum assay is suitable for use in epidemiologic research. Moreover, the high sensitivity of the assay and thus the ability to quantify concentrations among postmenopausal women and men, further supports that this assay may be suitable for studies of serum progesterone metabolite concentrations and risk of breast cancer or other hormonally-related cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, Division of Cancer Epidemiology and Genetics, National Cancer Institute. The authors gratefully acknowledge Norma Diaz-Mayoral, Gretchen Gierach, Barbara Fuhrman, and Cher Dallal for assistance with sample acquisition.

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Figure 1. Schematic of progesterone precursor pregnenolone, progesterone, and progesterone metabolites measured using HPLC-MS/MS assay and their hypothesized role in breast cancer risk.

Figure 2.

Among repeat samples from 7 men (Panel A) and 8 postmenopausal women (Panel B) results of assay reproducibility for pregnenolone, progesterone, 5 α dihydroprogesterone, 3 α dihydroprogesterone, and 20 α dihydroprogesterone, respectively. Serum concentrations of each hormone (ng/dL) on a log-scale (y-axis) plotted by batch (x-axis). Each symbol corresponds to a different subject. Duplicate readings from the same person have the same symbol, if values for the duplicate reading within a batch overlapped, only one symbol is shown.

Geometric mean concentration (ng/dL) and rank order of 5 progesterone-related hormones by sex/menopausal status.

Table 1

	Men			Premenopausal Women			Postmenopausal Women		
	GM	95% CI	Rank	GM	95% CI	Rank	GM	95% CI	Rank
Pregnenolone	28.0	(18.2–39.8)	1	35.4	(30.7–40.8)	3	14.2	(7.2–19.7)	1
Progesterone	4.2	(2.9–6.3)	3	63.4	(29.3–137.2)	1	3.4	(3.0–3.9)	4
3 α -dihydroprogesterone	0.7	(0.5–0.9)	5	1.4	(1.1–1.7)	5	0.7	(0.5–1.1)	5
5 α -dihydroprogesterone	6.8	(4.7–11.0)	2	25.2	(17.3–36.9)	4	6.3	(4.8–7.6)	2
20 α -dihydroprogesterone	3.9	(2.5–5.1)	4	35.8	(20.1–63.9)	2	4.0	(2.7–5.2)	3

Reproducibility of liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for 5 progesterone-related hormones by sex/menopausal status.

Table 2

	Men		Premenopausal Women		Postmenopausal Women	
	CV (%)	ICC (%)	CV (%)	ICC (%)	CV (%)	ICC (%)
Pregnenolone	1.7	99.9	1.9	99.8	2.1	99.9
Progesterone	2.5	99.8	1.6	100.0	2.1	99.7
3 α -dihydroprogesterone	1.3	99.8	2.4	99.9	2.5	99.9
5 α -dihydroprogesterone	1.7	99.8	1.7	100.0	1.9	99.8
20 α -dihydroprogesterone	1.9	99.9	1.2	100.0	2.7	99.7