

Reproducibility of Serum Sex Steroid Assays in Men by RIA and Mass Spectrometry

Ann W. Hsing,¹ Frank Z. Stanczyk,² Alain Bélanger,³ Paul Schroeder,⁴ Lilly Chang,² Roni T. Falk,¹ and Thomas R. Fears¹

¹Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland; ²Departments of Obstetrics and Gynecology and Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California; ³Oncology and Molecular Endocrinology Research Center, Quebec University Hospital Center and Laval University, Quebec, Canada; and ⁴Westat, Rockville, Maryland

Abstract

There is an increasing trend to apply gas chromatography combined with mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) assay methods to large-scale epidemiologic studies for the measurement of serum sex steroids. These methods are generally considered the gold standard for sex steroid measurements because of their accuracy, sensitivity, turnaround time, and ability to assess a more complete panel of steroid metabolites in the same run. In this report, we evaluated the precision, including within-batch (intra) and between-batch (inter) reproducibility, of steroid hormone measurements determined by GC-MS and LC-MS/MS assays and RIA and compared measurements among these methods. Specifically, 282 overnight fasting serum samples from 20 male volunteers were analyzed for 12 steroid metabolites by GC-MS or LC-MS/MS in one lab over a 4-month period. Six of the analytes were also measured by RIA in another lab. Unconjugated hormones, including testosterone, dihydrotestosterone, dehydroepiandrosterone, androstenedione, androst-5-ene-3 β ,17 β -diol, estrone, and estradiol, were measured by GC-MS, whereas conjugated hormones, including DHEA sulfate, androsterone glucuronide, 5 α -androstane-3 α ,17 β -diol 3-glucuronide, 5 α -androstane-3 α ,17 β -diol 17-glucuronide, and estrone sulfate, were measured by LC-MS/MS. A subset of these hormones, including testosterone, dihydrotestosterone, androstenedione, 5 α -androstane-3 α ,17 β -diol 17-glucuronide, estrone, and estradiol, were also measured by RIA following extraction and chromatography. We used the coefficient of varia-

tion (CV) and the intraclass correlation coefficient (ICC) to assess within- and between-batch assay variations. For the 12 analytes measured by GC-MS or LC-MS/MS, CVs and ICCs for within- and between-batch measurements were similar, with CVs ranging from 6.1% to 21.4% and ICCs ranging from 87.6% to 99.2%. The six analytes measured by RIA had good CVs and ICCs, with CVs <10% and ICCs >70% (range, 71.7–99.7%). For the six metabolites that were measured by both methods, the CVs were similar, whereas the ICCs were generally higher with the GC-MS method. The absolute values for each analyte measured by RIA and GC-MS differed, with RIAs usually yielding markedly higher levels than GC-MS, although the Pearson and Spearman correlation coefficients for these six analytes were near one and all were significant ($P < 0.001$). Our results show that RIA, GC-MS, and LC-MS/MS assays for androgens and estrogens in the two labs included in the study have good reproducibility, as measured by small CVs (<15%) and high ICCs (>80%), with the exception of estradiol (71.7%) when measured by RIA. Despite substantial differences in absolute measurements of sex steroid hormones by RIA and MS methods, correlations between the two assays for the six sex steroids measured in the two labs were high (>0.9). However, it is important for future large epidemiologic studies to incorporate MS with high reproducibility and specificity to measure a more complete profile of androgen and estrogen metabolites to clarify the role of sex steroids in prostate cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(5):1004–8)

Introduction

Although abundant biological data indicate that endogenous androgens play an important role in the development of prostate cancer, epidemiologic data from human studies are inconclusive (1–6). The inconsistencies in these epidemiologic investigations are due, in part, to methodologic limitations, including intra- and inter-subject and inter-laboratory variations. In many epidemiologic studies, the coefficients of variation (CV) of steroid hormone assays are sometimes larger than inter-subject variation, making it difficult to detect small (<15%) case-control differences.

Most epidemiologic studies use radioimmunoassay (RIAs) to measure sex steroid hormones because they have acceptable turnaround times and are relatively inexpensive. Precision and accuracy of certain RIAs, especially direct assay without extraction, are of concern in some studies (7–18). Gas or liquid chromatography combined with mass spectrometry (GC-MS or LC-MS/MS) are considered the gold standard methods for sex steroid hormone quantification. These methods, detecting the molecular mass of the analyte, are generally more sensitive and allow the simultaneous detection of multiple analytes in the same assay. They have not been widely used in epidemiologic studies to measure circulating levels of hormones because, historically, these assays have required large volumes of serum and have been labor-intensive and costly. Recently, methods have been developed that use the capability of either GC or high-performance liquid chromatography that is linked to a mass detector with multiple reaction monitoring (19). These new methods require much less serum for the same panel of hormones than standard RIAs, are relatively high throughput, and have good potential for large-scale epidemiologic studies. Thus, we conducted a study to evaluate the reproducibility of

Received 9/18/06; revised 2/2/07; accepted 2/20/07.

Grant support: Intramural Research Program of the NIH, National Cancer Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ann W. Hsing, Division of Cancer Epidemiology and Genetics, National Cancer Institute, EPS 5024, MSC 7234, 6120 Executive Boulevard, Bethesda, MD 20852-7234. Phone: 301-435-3980; Fax: 301-402-0916. E-mail: hsinga@mail.nih.gov

Copyright © 2007 American Association for Cancer Research.

doi:10.1158/1055-9965.EPI-06-0792

high-throughput MS assays and compare their values with those derived from RIAs.

Materials and Methods

The design of the study is depicted in Fig. 1. To summarize, overnight fasting sera from 20 male volunteers were obtained to measure 12 steroids by GC-MS or LC-MS/MS and 6 steroids by RIA. At the MS lab, unconjugated hormones, including testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (4-Dione), androst-5-ene-3 β ,17 β -diol (A5-diol), estrone (E₁), and estradiol (E₂), were measured by GC-MS, and conjugated hormones, including DHEA sulfate (DHEAS), androsterone glucuronide (ADT-G), 5 α -androstane-3 α ,17 β -diol 3-glucuronide (3 α -diol-3G), 5 α -androstane-3 α ,17 β -diol 17-glucuronide (3 α -diol-17G, also known as AAG), and estrone sulfate (E₁S), were measured by LC-MS/MS. The assays for 3 α -diol-3G and 3 α -diol-17G were developed because two different UDP-glucuronosyltransferase 2B (UGT2B) proteins, UGT2B7 and UGT2B15, are responsible for their production (20). At the RIA lab, we measured a subset of these steroids, including testosterone, DHT, 4-Dione, 3 α -diol-17G, E₁, and E₂.

Sample Collection

Blood Collection. For the reproducibility assessment, we collected 85 mL of overnight fasting blood from 20 male volunteers between the ages of 50 and 65 years. Subjects with a history of cancer or heart disease, or who were currently taking hormone supplements, were excluded. Up to 10 red-topped vacutainers (no additive) were collected, each containing 8.5 mL of blood. Some subjects were unable to give 10 vacutainers of blood for various reasons, including a few subjects who experienced lightheadedness or an extremely slow blood draw. Once collected and labeled, the blood was separated at the National Cancer Institute contracting lab within 6 h.

Aliquoting. For each subject, all vacutainers were spun at the same time at $900 \times g$ for 10 min. The serum for each subject was combined to produce a uniform pool. Aliquots of 1.5 mL of serum were used for the GC-MS and LC-MS/MS assays and 2.5 mL of serum for the RIA assays. Because we were sending two samples per subject within each batch and a total of four batches to both labs for reproducibility assessment, a total of 32 mL of serum was required for each subject. For subjects who had <32 mL serum, the samples destined for GC-MS or LC-MS/MS assays were aliquoted first, and the remaining serum

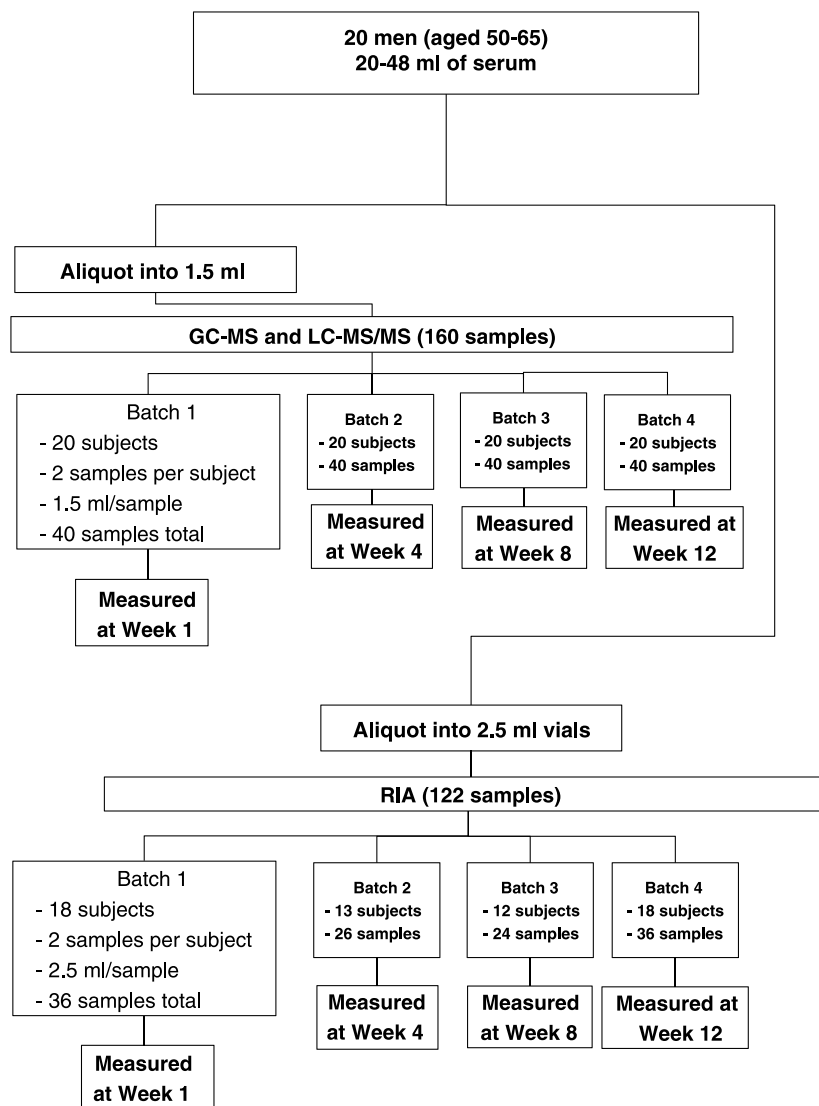


Figure 1. Study design.

was divided among batches destined for the RIA assays. Samples were stored at -70°C at the National Cancer Institute repository until assayed.

Batching of Specimens. To assess both intra-batch and overall (inter-batch) variation, we included two aliquots per subject in each of the four batches for each laboratory and sent the samples to the labs at four different times about 4 weeks apart (Fig. 1). For the GC-MS and LC-MS/MS assays, each batch had two samples from each of the 20 subjects, for a total of 40 samples. For the same subject, there were eight identical samples in all four batches. In total, we sent 160 samples ($1.5\text{ mL} \times 160$) from 20 subjects for the GC-MS and LC-MS/MS assays. Aliquots in each batch were placed randomly. All samples were blinded and identified by specimen IDs only.

For the RIA assays, some subjects did not have sufficient serum for eight samples ($2.5\text{ mL} \times 8$). Thus, we included 18 subjects in the first batch, 13 in the second batch, 12 in the third batch, and 18 in the fourth batch. In total, we sent 122 samples from 20 subjects to the RIA lab. Of these four batches, there were 12 subjects who had eight identical samples (two in each batch) across the four batches.

Steroid Assays

RIA Method. Testosterone, DHT, 4-Dione, 3α -diol-17G, E_1 , and E_2 were quantified in serum as previously described (21, 22). With the exception of 3α -diol-G, the analytes were quantified following organic solvent extraction and Celite column partition chromatography, using ethylene glycol as the stationary phase. Before the purification steps, a known amount of the appropriate internal standard ($^3\text{H-T}$, $^3\text{H-DHT}$, $^3\text{H-A4}$, $^3\text{H-E}$, or $^3\text{H-E}_2$) was added to each sample to follow procedural losses. 4-Dione and T were processed using 0.2 mL of serum and were eluted off the column with 0% and 35% toluene in isooctane, respectively. DHT, E_1 , and E_2 were analyzed in 0.8 mL of serum. DHT was eluted with 10% toluene in isooctane, whereas E_1 and E_2 were eluted with 15% and 40% ethyl acetate in isooctane, respectively. The conjugated steroid, 3α -diol-G, was quantified by direct RIA (23). For 3α -diol-G, no purification steps were used before the RIA because the antiserum in this RIA is highly specific. The sensitivities of the RIAs are as follows: 0.035 ng/mL for 4-Dione, 0.02 ng/mL for T, 0.02 ng/mL for DHT, 5 pg/mL for E_1 , 3 pg/mL for E_2 , and 0.5 ng/mL for 3α -diol-G.

GC-MS and LC-MS/MS Methods. Unconjugated steroids were measured by GC-MS using an aliquot of 0.75 mL for the analysis of T, DHT, DHEA, 4-Dione, A5-diol, E_1 , and E_2 . Internal standards were added to follow procedural losses. A liquid/liquid extraction was followed by silica gel chromatography, derivatization of the analytes, and liquid/liquid extraction. The residue was then submitted to GC using a 30-m column, and the analytes were detected at the molecular mass of the steroid derivative using single-ion monitoring. For conjugated steroids, an aliquot of 0.5 mL was used to measure ADT-G, 3α -diol-3G, and 3α -diol-17G by LC-MS/MS, and a second aliquot of 0.1 mL was used for the analysis of DHEAS and $E_1\text{S}$. The technique used C18 solid-phase extraction (Sep-Pack column), and the extract was injected into a high-performance liquid chromatography unit that was coupled to a mass detector using multiple-reaction monitoring (parent and daughter ions). Sensitivities of the MS assays are as follows: T, 0.02 ng/mL; DHT, 8 pg/mL; DHEA, 0.05 ng/mL; A5-diol, 0.05 ng/mL; 4-Dione, 0.01 ng/mL; E_1 , 8 pg/mL; E_2 , 2 pg/mL; ADT-G, 0.6 ng/mL; 3α -diol-3G, 0.5 ng/mL; DHEAS, 0.08 $\mu\text{g/mL}$, $E_1\text{S}$, 0.075 ng/mL.

Statistical Analysis. For each of the hormones, a nested components-of-variance analysis was done using measurements on the natural logarithmic scale. Variance components

Table 1. CVs and ICCs for hormones measured by MS and RIA

| Serum analyte | MS | | | | RIA | | | |
|---------------------|--------------|------|----------|------|--------------|------|----------|------|
| | Within-batch | | Overall* | | Within-batch | | Overall* | |
| | CV | ICC | CV | ICC | CV | ICC | CV | ICC |
| Androgen | | | | | | | | |
| Testosterone | 6.2 | 94.8 | 6.2 | 94.8 | 7.2 | 91.3 | 8.5 | 88.2 |
| DHT | 6.1 | 95.8 | 6.1 | 95.8 | 7.2 | 91.4 | 9.1 | 86.7 |
| DHEA | 15.3 | 94.3 | 16.0 | 93.8 | — | — | — | — |
| DHEAS | 20.2 | 92.7 | 21.4 | 91.9 | — | — | — | — |
| ADT-G | 6.4 | 99.2 | 7.4 | 98.8 | — | — | — | — |
| 4-Dione | 10.6 | 89.4 | 11.2 | 88.2 | 6.9 | 95.2 | 9.9 | 90.6 |
| A5-diol | 13.6 | 91.7 | 14.4 | 90.8 | — | — | — | — |
| 3α -diol-3G | 7.5 | 98.3 | 7.9 | 98.2 | — | — | — | — |
| 3α -diol-17G | 6.7 | 98.6 | 6.9 | 98.6 | 2.9 | 99.7 | 5.9 | 98.9 |
| Estrogen | | | | | | | | |
| E_1 | 7.4 | 93.5 | 9.8 | 89.2 | 8.3 | 90.5 | 9.8 | 87.2 |
| $E_1\text{S}$ | 15.9 | 95.3 | 15.9 | 95.3 | — | — | — | — |
| E_2 | 7.0 | 89.9 | 7.9 | 87.6 | 6.6 | 84.3 | 9.6 | 71.7 |

*Includes both within- and between-batch variation.

were estimated for subjects, batches within subject, and aliquots within batch. We used two measures of reproducibility derived from these variance components (9).

The common measure of reproducibility is the overall CV that is appropriate when study samples are completely randomized. The sum of the components associated with batch and aliquot is an estimate of the square of the overall CV. The within-batch CV is similarly estimated using only the aliquots within the batch component. The within-batch CV is appropriate when matched case-control study samples are in the same batch.

The intraclass correlation (ICC) is estimated by the ratio of the component associated with subjects and the sum of all components. The ICC is of importance to the epidemiologist because it indicates the effect of measurement error on study results. Specifically, regression analyses relating the log relative risk of disease to the log hormone assay level will be attenuated by the ICC.

In our experience, the most useful assays have CVs that are <20% and ICCs that are >80%. Pearson and Spearman correlation coefficients were used to measure the correlation of the measurements between the RIA and GC-MS assay methods.

Results

Table 1 shows the within and between-batches CVs and ICCs for the steroids measured by MS and RIA. The six analytes measured by RIA had good CVs and ICCs, with all CVs <10% and ICCs >80%. For both methods, CVs for between batch were only slightly larger than those for within batches, whereas between-batch ICCs were somewhat lower than those for within batches. For the 12 analytes measured by MS, CVs for within- and between-batch measurements were similar, ranging from 6.1% to 21.4%, with DHEAS having the largest CVs (20.2% and 21.4%). ICCs for the MS assays ranged from 87.6% to 99.2%, with E_2 having the lowest ICC (87.6%). For the six metabolites that were measured at both labs, CVs were quite similar, whereas the ICCs were generally larger in the MS lab, particularly for E_2 .

Table 2 shows the absolute values, including means and standard deviations (SDs), for each analyte in each batch assayed by RIA and MS. As shown, the absolute levels measured by RIA and MS were quite different, with RIA consistently yielding significantly higher levels than MS for each metabolite. Pearson and Spearman correlation coefficients for six analytes that were measured by both RIA and CC MS were near one and all were significant ($P < 0.001$).

Table 2. Means and SDs of serum hormones measured in four batches by RIA and MS

| Hormones | RIA | | | | | MS | | | | |
|--------------------------|-------------|-------------|-------------|-------------|--------------------------|-------------|-------------|-------------|-------------|--------------------------|
| | Batch | | | | All batches (n = 122) | Batch | | | | All batches (n = 160) |
| | 1 (n = 36) | 2 (n = 26) | 3 (n = 24) | 4 (n = 36) | | 1 (n = 40) | 2 (n = 40) | 3 (n = 40) | 4 (n = 40) | |
| Androgen | | | | | | | | | | |
| Testosterone (ng/mL) | 4.21 (1.19) | 4.42 (0.97) | 4.28 (1.04) | 4.66 (1.18) | 4.39 (1.09) | 3.68 (0.92) | 3.92 (1.09) | 3.80 (1.03) | 3.87 (1.02) | 3.82 (1.00) |
| DHT (ng/mL) | 0.43 (0.11) | 0.42 (0.09) | 0.47 (0.10) | 0.46 (0.12) | 0.44 (0.10) | 0.32 (0.09) | 0.34 (0.09) | 0.34 (0.09) | 0.33 (0.09) | 0.33 (0.09) |
| 4-Dione (ng/mL) | 0.90 (0.28) | 0.97 (0.31) | 0.91 (0.30) | 0.95 (0.34) | 0.93 (0.30) | 0.77 (0.27) | 0.71 (0.24) | 0.76 (0.25) | 0.78 (0.26) | 0.76 (0.25) |
| DHEA (ng/mL) | — | — | — | — | — | 1.93 (1.10) | 2.07 (1.19) | 2.19 (1.26) | 2.29 (1.69) | 2.12 (1.31) |
| DHEAS (μg/mL) | — | — | — | — | — | 0.92 (0.62) | 0.86 (0.52) | 0.92 (0.60) | 0.85 (0.53) | 0.89 (0.55) |
| ADT-G (ng/mL) | — | — | — | — | — | 47.3 (35.9) | 45.1 (34.2) | 44.9 (34.4) | 43.3 (33.8) | 45.1 (33.9) |
| A5-diol (ng/mL) | — | — | — | — | — | 0.66 (0.26) | 0.72 (0.28) | 0.67 (0.31) | 0.72 (0.29) | 0.69 (0.28) |
| 3α-diol-3G (ng/mL) | — | — | — | — | — | 1.99 (1.24) | 1.95 (1.25) | 1.92 (1.25) | 1.95 (1.28) | 1.95 (1.23) |
| 3α-diol-17G (ng/mL) | 8.32 (4.27) | 8.02 (4.13) | 7.53 (3.92) | 7.59 (3.82) | 7.86 (3.97) | 4.65 (2.73) | 4.39 (2.74) | 4.33 (2.70) | 4.40 (2.76) | 4.44 (2.68) |
| Estrogen | | | | | | | | | | |
| E ₁ (pg/mL) | 44 (12) | 45 (12.8) | 41 (12) | 41 (15) | 43 (13) | 31 (10) | 29 (11) | 31 (10) | 34 (11) | 31 (11) |
| E ₁ S (ng/mL) | — | — | — | — | — | 0.6 (0.5) | 0.7 (0.6) | 0.7 (0.6) | 0.7 (0.6) | 0.7 (0.6) |
| E ₂ (pg/mL) | 34 (5) | 33 (6.41) | 36 (6) | 30 (5) | 33 (6) | 20 (5) | 22 (5) | 21 (5) | 22 (5) | 21 (5) |

NOTE: Correlation of measurements in all batches between RIA and MS.

Discussion

In this study of 282 samples, we showed that RIA and MS have good reproducibility for all the sex steroid hormones assayed, despite the low concentrations of estrogens in men, with the exception of RIA measurements of estradiol. However, the absolute concentrations were significantly higher with RIA for most of the steroids, particularly E₂, indicating the possibility of detecting immunoreactive materials through cross-reaction with RIA. Although the absolute levels of sex steroid hormones measured by these two methods differed, the correlations between the two methods were high (>0.9), suggesting that when ranks or quartiles are used to categorize hormone levels in subjects for assessing the relationship between serum hormones and cancer, measurements from these two methods are likely to classify subjects into the same quartile, thereby yielding similar risk estimates. However, the absolute difference in serum levels of sex hormones between cases and controls in epidemiologic studies may differ, depending on the assay methods and antibodies used. Thus, it is important when using RIA to consider assay methods when comparing absolute values directly across studies.

CVs for within-batch and overall (between-batch) assays for both methods were generally satisfactory, except for the four analytes (DHEA, DHEAS, A5-diol, and E₁S) measured by MS. For these hormones, CVs were over 15%. It is not surprising that the CVs for between-batch assays from both RIA and MS were larger than those for within-batch assays and are probably due to an accumulation of small differences between assays over time (4 months), including differences in reagents, technicians, minor day-to-day variation in sample processing, use of an internal standard to follow losses in each batch, and preparation of the standard curve for each batch.

The absolute measurements of each analyte in the study were within the expected range (as established by RIA), whereas the values obtained by MS were significantly lower. The reasons for these differences are likely due to the lack of specificity of RIA compared with GC-MS and LC-MS/MS assays, which are usually considered highly specific due to the high resolution of compounds by GC and LC and the high specificity and sensitivity of MS.

In conclusion, results from this study show a high correlation between steroid hormone levels measured by RIA and MS despite the significant differences in absolute measurements. The high correlations between RIA and MS suggest that these two methods are likely to yield similar risk estimates in epidemiologic studies when quartiles are used for analysis. However, it is important for future large-scale epidemiologic

studies to incorporate MS with high reproducibility, accuracy, and specificity to measure a more complete profile of androgen and estrogen metabolites to clarify further the role of sex steroids in prostate cancer.

Acknowledgments

We thank Karen Stewart of Westat for help with study management and Rene Bérubé and Patrick Bélanger of Laval University, Quebec, Canada for MS assays.

References

- Hsing AW, Reichardt JK, Stanczyk FZ. Hormones and prostate cancer: current perspectives and future directions. *Prostate* 2002;52:213–35.
- Hsing AW. Hormones and prostate cancer: what's next? *Epidemiol Rev* 2001;23:42–58.
- Eaton NE, Reeves GK, Appleby PN, Key TJ. Endogenous sex hormones and prostate cancer: a quantitative review of prospective studies. *Br J Cancer* 1999;80:930–4.
- Hsing AW, Chokkalingam AP. Prostate cancer epidemiology. *Front Biosci* 2006;11:1388–413.
- Platz EA, Giovannucci E. The epidemiology of sex steroid hormones and their signaling and metabolic pathways in the etiology of prostate cancer. *J Steroid Biochem Mol Biol* 2004;92:237–53.
- Gann PH, Hennekens CH, Ma J, Longcope C, Stampfer MJ. Prospective study of sex hormone levels and risk of prostate cancer. *J Natl Cancer Inst* 1996;88:1118–26.
- Toniolo P, Koening KL, Pasternack BS, et al. Reliability of measurements of total, protein-bound, and unbound estradiol in serum. *Cancer Epidemiol Biomarkers Prev* 1994;3:47–50.
- Rinaldi S, Dechaud H, Biessy C, et al. Reliability and validity of commercially available, direct radioimmunoassay for measurement of blood androgens and estrogens in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 2001;10:757–65.
- Fears TR, Ziegler RG, Donaldson JL, et al. Reproducibility studies and interlaboratory concordance for androgen assays in female plasma. *Cancer Epidemiol Biomarkers Prev* 2000;9:403–12.
- Dorgan JF, Fears TR, McMahon RP, et al. Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. *Steroids* 2002;67:151–8.
- Gail MH, Fears TR, Hoover RN, et al. Reproducibility studies and interlaboratory concordance for assays of serum hormone levels: estrone, estradiol, estrone sulfate, and progesterone. *Cancer Epidemiol Biomarkers Prev* 1996;5:835–44.
- McShane LM, Dorgan JF, Greenhut S, Damato JJ. Reliability and validity of serum sex hormone measurements. *Cancer Epidemiol Biomarkers Prev* 1996;5:923–8.
- Michaud DS, Manson JE, Spiegelman D, et al. Reproducibility of plasma and urinary sex hormone levels in premenopausal women over a one-year period. *Cancer Epidemiol Biomarkers Prev* 1999;8:1059–64.
- Shibata A, Lee MM, Meyer PB. Laboratory assay reproducibility of serum estrogens in umbilical cord blood samples. *Cancer Epidemiol Biomarkers Prev* 1999;8:147–51.
- Potischman N, Falk RT, Laiming VA, Sitteri PK, Hoover RN. Reproducibility of laboratory assays for steroid hormones and sex hormone-binding globulin. *Cancer Res* 1994;54:5363–7.

16. Hankinson SE, Manson JE, London SJ, Willett WC, Speizer FE. Laboratory reproducibility of endogenous hormone levels in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 1994;3:51–6.
17. Falk RT, Dorgan JF, Kahle L, Potischman N, Longcope C. Assay reproducibility of hormone measurements in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 1997;6:429–32.
18. Falk RT, Gail MH, Fears TR, et al. Reproducibility and validity of radioimmunoassays for urinary hormones and metabolites in pre- and postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 1999;8: 567–77.
19. Xu X, Ziegler RG, Waterhouse DJ, Saavedra JE, Keefe LK. Stable isotope dilution high-performance liquid chromatography-electrospray ionization mass spectrometry method for endogenous 2- and 4-hydroxyestrones in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;780: 315–30.
20. Belanger A, Pelletier G, Labrie F, Barbier O, Chouinard S. Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. *Trends Endocrinol Metab* 2003;14:473–9.
21. Goebelsmann U, Bernstein GS, Gale JA, et al. Serum gonadotropin testosterone estradiol and estrone levels prior to and following bilateral vasectomy. In: Lepow IH, Crozier R, editors. *Vasectomy: immunologic and pathophysiologic effects in animals and man*. New York: Academic Press; 1979. p. 165–75.
22. Goebelsmann U, Horton R, Mestman JH, et al. Male pseudohermaphroditism due to testicular 17-hydroxysteroid dehydrogenase deficiency. *J Clin Endocrinol Metab* 1973;36:867.
23. Narang R, Rao J, Savjani G, Peterson J, Gentschein E, Stanczyk FZ. radioimmunoassay kit for the quantitative measurement of androstanediol glucuronide in unextracted serum. Presented at the 17th National Meeting of the Clinical Ligand Assay Society, April 10–13, 1991, Chicago, IL, Poster #12.