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METHODOLOGICAL NOTE

Biomarkers (sex-hormone binding globulin (SHBG), bioavailable oestradiol, and bioavailable testosterone) and processing of blood samples in epidemiological studies

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Introduction

Decisions about the type of blood specimen collection tube [EDTA (ethylenediaminetetraacetic acid) plasma, heparinized plasma, or serum] generally depend on the requirements for specific biological markers identified at the beginning of the study. By the time the specimens are analysed, techniques for analysing may have changed, and new hypotheses may suggest new biomarkers or ones not previously suspected to be involved in the etiologic pathway. Different biological markers may have different optimal initial processing.

In the 1980s we conducted a population-based case-control study of a hormone-related cancer taking blood samples for assaying hormonal biomarkers. We chose EDTA plasma tubes for blood collection because of the advantages in assaying lipids. Prior to assaying the samples from our case-control study, we assessed the reproducibility of SHBG and bioavailable oestradiol and testosterone assays in EDTA plasma in three laboratories.

Methods

Three established endocrine laboratories were sent four batches of EDTA plasma (frozen at –70 °C). Each batch contained randomized, blinded, duplicate samples from 5 men and 15 women. The

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laboratories were asked to assay each batch for 17 analytes, including SHBG, bioavailable testosterone for men, and bioavailable oestradiol for women once during each of four consecutive months. Each laboratory was asked to use their standard procedures and to choose the order of the 17 assays. Duration of thawing and number of freeze–thaw cycles were not specified nor monitored.

To determine SHBG, laboratories 1 and 3 used DSL (Diagnostic Systems Laboratory) kits and laboratory 2 performed a plasma testosterone binding capacity assay. Bioavailable oestradiol and bioavailable testosterone were determined by similar methods (Sodergard *et al*. 1982) in all laboratories.

Since CVs for SHBG were unacceptably high in the 'initial' study, a second, 'accelerated' SHBG reproducibility study was done using laboratory 1 to evaluate more stringent specifications for handling samples. In this study, frozen EDTA plasma from two mid-luteal and three postmenopausal women were assayed. Ten batches of randomized, blinded, duplicate samples were sent, with one batch assayed on each of ten consecutive working days. The samples in this study were thawed once, 10 min before assaying.

Statistical methods

Results were analysed on the natural logarithmic scale to stabilize variance. For each subject group, a nested components of variance analysis was performed. Components of variance were estimated for batch and aliquots in the same batch. Estimates of the variance components were obtained by restricted maximum likelihood and used to estimate the CV (Gail *et al*. 1996). This CV incorporates the batch-to-batch variation and may be much larger than the usual laboratory CV which is based only on measurement variability within a single batch. Geometric means reduce the effect of a few extreme values.

Results and discussion

Bioavailable oestradiol

The mean bioavailable oestradiol (E2) concentrations were highest in laboratory 3 and similar in laboratories 1 and 2 (table 1). The estimated CVs for the bioavailable oestradiol assay ranged from 11% to 26%.

Bioavailable testosterone

The mean bioavailable testosterone concentrations were similar in the three laboratories. The estimated CVs were 29% and 38% for laboratory 1 and 3, respectively, and only 16% for laboratory 2 (table 1).

SHBG

The SHBG measurements were lowest in laboratory 1 and similar in laboratories 2 and 3. All estimated CVs were unacceptably high. The CVs ranges for samples were 25% to 75% (table 1).

The ten-day accelerated study

This study evaluated the effect of stringent handling specification. The results from this second study showed much smaller coefficients of variations: 8.1% for mid-luteal phase and 8.4% for postmenopausal women (table 1).

In our experience laboratory CVs less than 10% are very good, though most CVs are in the 15–20% range (Gail *et al*. 1996, Falk 1999). The CVs for SHBG in the initial study were poor (24–75%) and the batch-to-batch variation was the largest component. The poor reliability was hypothesized to be related to either the amount of time the samples spent at temperatures above freezing while assays for 17 analytes were done each month or the instability of the kit over time.

Table 1. Estimated coefficients of variation for bioavailable oestradiol, bioavailable testosterone, and SHBG, by laboratory.

Gender and menstrual status	Laboratory 1		Laboratory 2		Laboratory 3		Accelerated study	
	Mean[a]	CV(%)	Mean	CV(%)	Mean	CV(%)	Mean	CV(%)
	Bioavailable oestradiol (pg ml^{-1})							
Women:								
Mid-follicular phase	63.1	15.4	69.8	15.4	84.9	20.0		
Mid-luteal phase	78.9	22.6	86.1	10.6	90.9	26.1		
Postmenopausal	4.5	18.9	4.9	17.2	10.7	24.5		
	Bioavailable testosterone (pg ml^{-1})							
Men	177.8	29.0	194.2	15.8	185.7	38.0		
	SHBG (nM l^{-1})							
Women:								
Mid-follicular phase	25.5	57.2	32.0	33.5	28.6	75.0		
Mid-luteal phase	38.5	37.3	42.8	23.9	45.5	71.0	24.0[b]	8.1
Postmenopausal	24.6	24.4	28.8	32.0	29.7	67.1	45.7[b]	8.4
Men	16.1	35.4	25.6	70.9	26.0	67.5		

[a] All means are geometric means.
[b] The means of the accelerated study can not be compared with the means of the initial study since different women's plasmas were used.

Biochemical journals report SHBG temperature sensitivity in a calcium-free medium (Rosner *et al*. 1974, Bocchinfuso and Hammond 1994). Since EDTA chelates calcium, it can remove calcium from SHBG. The longer an EDTA plasma is at room temperature, or even in the refrigerator, the more likely the SHBG is denatured (personal communication, D. Walt Chandler, Endocrine Sciences Laboratory, March 10, 1998). The resulting dimer destabilization could result in loss of steroid binding causing inappropriately low measurements of SHBG steroid binding capacity or of SHBG measured by immunoassay (Bocchinfuso and Hammond 1994) and cause an increase in the fraction of oestradiol and testosterone not bound to SHBG. Accordingly, in this study bioavailable oestradiol and testosterone concentrations also had high CVs. The total oestradiol and testosterone concentrations (Gail *et al*. 1996) had better CVs, hence the subtracted hormone bound to SHBG in bioavailable calculations must be the problem.

To the best of our knowledge, the literature has no study of the reproducibility of SHBG measurements in EDTA plasma. However, reproducibility studies with *serum* samples have reported total CVs (including within and between batch variation) of 6–13 % in one study (McShane *et al*. 1996) and 6–19 % in another with only within-batch CVs (Hankinson *et al*. 1994).

In the initial study, the means of these three measurements for the same specific gender/menstrual status subgroups vary by 6–237 % among the laboratories. Differences even existed between laboratories using the same assay kit (SHBG in laboratories 1 and 3), suggesting specimen handling differences. In this study storage conditions for the EDTA plasma samples were not carefully monitored by the laboratories, however, in the second, accelerated reproducibility study when samples were carefully handled with only a single thawing, it was possible to have reproducible measurements of SHBG in EDTA plasma. This study, conducted in

14 days, did not determine if instability of the SHBG assay kits over 3 months also contributed to the poor CVs. Since other epidemiologists assaying for biomarkers from previously collected biological samples may encounter problems of less than optimal initial processing, we recommend tightly controlled laboratory handling of all study specimens with minimal intervals between batches. We also recommend *a priori* reproducibility studies of assays on similarly processed samples.

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