CEBP Focus: Biorepository and Biospecimen Science

Collection, Processing, and Storage of Biological Samples in Epidemiologic Studies: Sex Hormones, Carotenoids, Inflammatory Markers, and Proteomics as Examples

Shelley S. Tworoger[1,2] and Susan E. Hankinson[1,2]

[1]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School; and
[2]Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts

Abstract

The measurement of biomarkers in blood specimens has become an integral component of many epidemiologic studies and introduces several decision points about specimen collection, processing, and storage for the investigator. We briefly discuss the current state of knowledge for four commonly assessed biomarkers: estrogens and other sex hormones, ascorbic acid and carotenoids, cytokines involved in the inflammatory response, and proteomics. Sex hormones are relatively robust to type of sample collected, delayed processing (if chilled), and long-term storage at <−70°C. Ascorbic acid and carotenoids also are relatively robust to sample type and delayed processing (if chilled); however, the blood sample should not be exposed to sunlight and must be stored at <−70°C to prevent substantial degradation. If ascorbic acid is of primary interest, an acid stabilizer should be added during processing. Less is known for cytokines and proteomics, although initial research suggests that these assays are sensitive to varying collection, processing, and storage methods. Overall, we recommend conducting pilot studies if any nonstandard collection, processing, or storage procedure is used. Finally, decisions about these issues depend primarily on the scientific questions of most interest, cost, flexibility, and resources. (Cancer Epidemiol Biomarkers Prev 2006;15(9):1578–81)

Introduction

The measurement of biomarkers in blood specimens has become an integral component of many epidemiologic studies. Use of blood biomarkers introduces several decision points at the study design phase for the investigator (1). We briefly will discuss specimen collection, processing, and storage for four blood biomarkers of broad interest to the epidemiologic community: estrogens and other sex hormones, ascorbic acid and carotenoids, cytokines involved in the inflammatory response, and proteomics. This will provide guidance for investigators interested in one or more of these markers and illustrate the range of information available and ongoing research needs. Substantial data have accrued for the sex steroids and carotenoids. Although increasing information is available for a subset of inflammatory markers (e.g., interleukin-6 and tumor necrosis factor-α), limited data are available for other markers of inflammation as well as for proteomics. Please note that, in the interest of space, we only cite representative references.

Sample Type and Timing of Collection

Either serum or plasma (with different anticoagulants) can be collected, each having advantages and disadvantages depending on the biomarker of interest (1). In addition, timing of the sample collection is important because some biomarkers can fluctuate yearly, seasonally, monthly, daily, or hourly.

Sex Hormones. In general, sex hormone levels (2-4) are similar when comparing serum, EDTA plasma, and heparinized plasma. Several (2, 4) studies have suggested higher levels of estradiol and testosterone in EDTA or heparinized plasma versus serum; however, differences were modest, and thus, both serum and plasma are acceptable for these biomarkers. One study (3) reported lower testosterone levels in citrate plasma versus serum that is likely due to a dilutional effect of the liquid anticoagulant (5).

Timing the sample collection for measurement of estrogen and progesterone is difficult in premenopausal women, as levels vary widely across the menstrual cycle. To date, several different strategies have been used. For example, in the Nurses' Health Study II, premenopausal women were asked to collect their samples at specific times in the menstrual cycle (early follicular and midluteal), when sex hormones are relatively stable from day to day, and return a postcard with the date of their next menstrual cycle. This allowed accurate dating of the luteal phase collection. Alternatively, the European Prospective Investigation into Cancer and Nutrition Study asked women to collect a sample on any day of their menstrual cycle and then provide specific dates of their cycle before (and if possible after) blood collection and their average cycle length (6). Ovulation kits also can be used to time sample collection for a certain number of days after ovulation. The method of collection is in part dependent on the study population and resources. In addition, a strong circadian variation exists for some steroids (e.g., dehydroepiandrosterone) and should be considered.

Received 5/3/06; accepted 6/16/06.
Grant support: NIH grants P01 CA87969 and CA49449.
Note: This paper is one of a series of articles that were presented at a methods workshop, "Sample Collection, Processing, and Storage for Large-Scale Studies: Biorepositories to Support Cancer Research," held during the AACR 97th Annual Meeting in 2006.
Requests for reprints: Susan E. Hankinson, Channing Laboratory, 181 Longwood Avenue, 3rd Floor, Boston, MA 02115. E-mail: nhseh@channing.harvard.edu

doi:10.1158/1055-9965.EPI-06-0629

Ascorbic Acid and Carotenoids. In general, carotenoid levels can be measured in either serum or plasma and are not affected by the anticoagulant used (7, 8). As for timing of collection, carotene and retinol levels can have substantial seasonal variations across the year (9), suggesting the importance of controlling for timing either in the study design or analyses.

Inflammatory Markers. Most studies to date have assessed interleukin-6 and tumor necrosis factor-α, and these cytokines are somewhat sensitive to specimen type (10, 11). However, results are conflicting about the relationship between type of plasma anticoagulant or serum used and measurable levels, although most studies suggest that EDTA plasma is optimal (10, 11). Clearly, more data are needed for a wider range of cytokines and about the best timing for blood collection for these biomarkers.

Proteomics. Several recent articles (12-14) suggest that serum samples provide a poor substrate for most proteomic assays because of the large number of serum-specific, clot-related peptides produced that account for >40% of all peptide peaks (13, 14). The best type of plasma anticoagulant varied by the type of proteomic assay being conducted (e.g., differential peptide display worked poorly with heparin plasma; ref. 14). Data are too limited to make specific recommendations about optimal timing of sample collection; thus, it is important to track all preanalytic variables when collecting samples, as some factors (e.g., fasting, time of day, and medication use) may affect the measured proteome.

Sample Processing

Sample collection and its subsequent processing should be conducted in a rigorous and standardized manner, and, although certain collection methods are advised (e.g., immediate processing of samples), they are sometimes not possible in epidemiologic studies. Alternate strategies, then, must be developed that are feasible and acceptable to participants and do not substantially affect assay measurement. For example, we wanted to collect blood samples from participants in the Nurses' Health Study II; however, because the cohort is geographically dispersed across the United States, it was infeasible to have samples collected at a central hospital or clinic. After pilot testing for feasibility, we mailed women a blood collection kit with instructions and asked them to have someone draw the blood and then ship it back via overnight courier and with a chill pack to the study laboratory where it was processed into plasma, RBC, and WBC components. Extensive pilot testing showed that many analytes were not substantially affected by the plasma remaining unprocessed for 24 to 48 hours, whereas others (e.g., vascular endothelial growth factor) cannot be assessed with this protocol. Delayed blood processing is a common occurrence in epidemiologic studies, and thus, substantial literature is available about its effects.

Sex Hormones. Delayed processing of up to 72 hours, either at room temperature or chilled, does not seem to alter levels of estrogens and most androgens (3, 4, 15-17), although several studies have reported increasing testosterone levels with delayed processing likely due to *ex vivo* conversion of precursor hormones (3, 16, 17). However, this increase was modest in magnitude and levels were highly correlated across delayed processing times, suggesting that it is acceptable to measure testosterone in samples where processing was delayed. Samples should be aliquoted into appropriate airtight tubes to prevent degradation with long-term freezing.

Ascorbic Acid and Carotenoids. Carotenoids are stable for up to 1 week in whole blood if kept chilled (16); at room temperature, some carotenoids can degrade, although the changes were small (17, 18). Delayed processing of >24 hours may degrade ascorbic acid levels even if the samples are chilled (3). The chemical structure of carotenoids and ascorbic acid makes them potentially susceptible to oxidative damage or isomerization with exposure oxygen, light, or heat (18). Several studies, however, have reported that exposure to indoor light, but not outdoor light, before processing or replacing air in vials with nitrogen gas did not alter levels substantially (18, 19), suggesting that normal processing methods are acceptable for carotenoids and ascorbic acid. If ascorbic acid is of primary interest, an acid stabilizer, such as metaphosphoric acid, should be included before freezing, although a recent study reported that, even after 7 to 11 years of storage at −196°C, ascorbic acid levels were reasonably well correlated ($r = \sim 0.6$) in samples with versus without an acid stabilizer (20).

Inflammatory Markers. In general, samples should be kept refrigerated or cooled during processing because some inflammatory markers can degrade or increase at room temperature (11, 17). Both interleukin-6 and tumor necrosis factor-α seem to degrade after ~4 to 6 hours at room temperature (11).

Proteomics. In contrast to recommendations for inflammatory markers, samples to be used for proteomic assays should be processed immediately at room temperature [because cool temperatures can activate platelets and release peptides into the sample *ex vivo* (14)], aliquoted into airtight tubes, and frozen in liquid nitrogen or −70°C freezers (13). Any other processing methods should be pilot tested thoroughly. It also may be important to make the sample platelet poor by additional centrifugation or filtering (13, 14) to reduce the influence of highly abundant platelet-derived proteins (14). Further, recent evidence recommends consideration of a protease inhibitor(s) before freezing to reduce cleavage of proteins *ex vivo* (13), although this will vary according to the specific assay of interest (i.e., whole proteins versus peptides), and care should be taken in analyzing results because the molecular mass of protease inhibitors can overlap those in the proteome (13).

Freezer Temperature and Long-term Storage

Both mechanical and liquid nitrogen freezers are used in epidemiologic studies. Storage temperatures vary, with liquid nitrogen freezers ranging from −130°C to −196°C (depending on whether samples are in the liquid or vapor phase) and mechanical freezers between −20°C and −80°C. In a study of 15 mechanical freezers, the freezer display panel reported the temperature as −81°C to −74°C but the measured temperature varied substantially (−90°C to −43.5°C) in part by location within the freezer (21). Thus, liquid nitrogen freezers may be a better choice for very long-term storage of samples, such as in prospective studies, because they have a consistently lower temperature. Clearly, degradation over time is an important issue especially for samples stored for long periods; however, assessing whether degradation has occurred is complex (e.g., it can be difficult to separate out degradation due to long-term storage versus changes in the laboratory assay over time).

Sex Hormones. Storage at −20°C may not be acceptable for sex hormones (22, 23). In particular, sex hormone–binding globulin may dissociate from estradiol and testosterone, decreasing measurable nonbound levels of these hormones

(22). However, long-term storage at <−70°C seems to be acceptable for most sex hormones (2).

Ascorbic Acid and Carotenoids. Substantial decreases in carotenoid levels can occur when stored at −20°C for only 6 months (19, 24). One study reported a 15% decrease in measurable carotenoid levels at 6 months and a 97% decrease over 10 years (19). However, carotenoid levels in samples stored at <−70°C remained stable for up to 10 years (19, 25). As mentioned previously, long-term storage of ascorbic acid generally requires the addition of an acid stabilizer before freezing.

Inflammatory Markers and Proteomics. Few studies have examined the effect of long-term storage on inflammatory marker levels or proteomic assays. Thus, in general, it is recommended to keep specimens stored at <−70°C to help assure valid results. Several studies have examined the effects of multiple freeze-thaw cycles on these biomarkers and report sensitivity of some inflammatory markers (11) as well as the proteome (13); hence, freeze-thaw cycles should be avoided.

Conclusions

Acceptable collection and storage procedures for sex hormones, carotenoids, inflammatory markers, and proteomics are shown in Appendix A. In addition, we leave the reader with several general recommendations. First, it is important to balance optimal sample type/collection with flexibility. For example, even if serum is a better sample type for one analyte of interest, collecting plasma may be particularly useful because WBC and RBC can be more easily aliquoted and stored at the same time. Second, one should attempt to standardize important blood collection variables (e.g., month or time of day of blood collection) across participants or within matched groups of participants. Related to this, careful collection of preanalytic variables for each sample collected is critical. Third, to minimize the possibility of degradation, it is best to freeze samples at the coldest possible temperature especially if samples will be stored for a long time. As always, it is important to pilot test any nonstandard methods thoroughly. Ultimately, decisions about sample type, processing method, and storage depend primarily on the scientific questions of most interest, cost, flexibility, and resources. Each study may require different choices.

Appendix A. Sample collection and processing for the measurement of various blood biomarkers

Sex hormones
- Relatively robust to various sample collection or processing methods
- Use of serum or EDTA or heparin plasma is acceptable
- Collect an ample volume of sample, as the assays require up to 0.3-0.5 mL per hormone
- If possible, collect a fasting, morning sample, and in premenopausal women, standardize the collection time in the menstrual cycle
- Delayed processing up to 72 hours is acceptable
- Store samples in airtight vials at ≤−70°C

Ascorbic acid and carotenoids
- Relatively robust to various sample collection or processing methods
- Use of serum or heparin plasma is standard; EDTA plasma is acceptable
- Can be exposed to indoor light, but not sunlight
- Has some seasonal variation; therefore, if assessing changes over time, collect in the same month or control for month in the analysis
- Processing samples can be delayed up to 1 week, but samples should be chilled
- Store in airtight vials at ≤−70°C
- For storage over long periods, best to include an acid stabilizer (e.g., metaphosphoric acid) if measuring ascorbic acid
- Can assay after two or fewer freeze-thaw cycles

Markers of inflammation
- Relatively few cytokines evaluated to date
- Modestly robust to various sample collection or processing methods
- EDTA plasma is best sample type, although serum and heparin plasma are acceptable
- Delay in processing time should be minimized (<48 hours) and samples kept chilled; tumor necrosis factor-α and IFN-δ can only be measured in samples processed immediately
- Store in airtight vials at ≤−70°C
- May assay after up to six freeze-thaw cycles depending on the analyte

Proteomic assays
- Sensitive to various sample collection or processing conditions
- Serum not recommended as it can introduce clot-derived peptides; use of EDTA, heparin, or citrate plasma depends on application
- Best to process sample to make it platelet poor to reduce platelet-derived peptides; consider inclusion of protease inhibitor(s) to reduce *ex vivo* cleavage of proteins
- Minimize number of freeze-thaw cycles (<2)
- Currently recommended processing procedure
  - Remove platelets by centrifuging twice or by centrifuging followed by a filtration step at room temperature
  - Process immediately or within 1-2 hours, although longer delays may be acceptable depending on the application
  - Store in airtight vials
  - Store in liquid nitrogen or −80°C in high-quality freezer
  - Track all preanalytic variables

References

1. Holland NT, Smith MT, Eskenazi B, Bastaki M. Biological sample collection and processing for molecular epidemiological studies. Mutat Res 2003;543:217–34.
2. Bolelli G, Muti P, Micheli A, et al. Validity for epidemiological studies of long-term cryoconservation of steroid and protein hormones in serum and plasma. Cancer Epidemiol Biomarkers Prev 1995;4:509–13.
3. Key T, Oakes S, Davey G, et al. Stability of vitamins A, C, E, carotenoids, lipids, and testosterone in whole blood stored at 4 degrees C for 6 and 24 hours before separation of serum and plasma. Cancer Epidemiol Biomarkers Prev 1996;5:811–4.
4. Taieb J, Benattar C, Birr AS, Lindenbaum A, Frydman R, Olivennes F. Delayed assessment of serum and whole blood estradiol, progesterone, follicle-stimulating hormone, and luteinizing hormone kept at room temperature or refrigerated. Fertil Steril 2000;74:1053–4.
5. Palmer-Toy DE, Szczepiorkowski ZM, Shih V, Van Cott EM. Compatibility of the Abbott IMx homocysteine assay with citrate-anticoagulated plasma and stability of homocysteine in citrated whole blood. Clin Chem 2001;47:1704–7.
6. Kaaks R, Berrino F, Key T, et al. Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). J Natl Cancer Inst 2005;97:755–65.
7. Olmedilla-Alonso B, Granado-Lorencio F, Blanco-Navarro I. Carotenoids, retinol, and tocopherols in blood: comparability between serum and plasma (Li-heparin) values. Clin Biochem 2005;38:444–9.
8. Smith JC, Jr., Lewis S, Holbrook J, Seidel K, Rose A. Effect of heparin and citrate on measured concentrations of various analytes in plasma. Clin Chem 1987;33:814–6.
9. Cooney RV, Franke AA, Hankin JH, et al. Seasonal variations in plasma micronutrients and antioxidants. Cancer Epidemiol Biomarkers Prev 1995;4:207–15.
10. De Jongh R, Vranken J, Vundelinckx G, Bosmans E, Maes M, Heylen R. The effects of anticoagulation and processing on assays of IL-6, sIL-6R, sIL-2R, and soluble transferrin receptor. Cytokine 1997;9:696–701.
11. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-α, and leptin. Cytokine 2000;12:1712–6.
12. Haab BB, Geierstanger BH, Michailidis G, et al. Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: systematic variation between sample types and calibration of mass spectrometry data. Proteomics 2005;5:3278–91.
13. Rai AJ, Gelfand CA, Haywood BC, et al. HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. Proteomics 2005;5:3262–77.
14. Tammen H, Schulte I, Hess R, et al. Peptidomic analysis of human blood specimens: comparison between plasma specimens and serum by differential peptide display. Proteomics 2005;5:3414–22.

15. Ellis MJ, Livesey JH, Evans MJ. Hormone stability in human whole blood. Clin Biochem 2003;36:109–12.
16. Kristal AR, King IB, Albanes D, et al. Centralized blood processing for the selenium and vitamin E cancer prevention trial: effects of delayed processing on carotenoids, tocopherols, insulin-like growth factor-I, insulin-like growth factor binding protein 3, steroid hormones, and lymphocyte viability. Cancer Epidemiol Biomarkers Prev 2005;14:727–30.
17. Hankinson SE, London SJ, Chute CG, et al. Effect of transport conditions on the stability of biochemical markers in blood. Clin Chem 1989;35:2313–6.
18. Clark S, Youngman LD, Chukwurah B, et al. Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days: implications for epidemiological studies. Int J Epidemiol 2004;33:518–25.
19. Mathews-Roth MM, Stampfer MJ. Some factors affecting determination of carotenoids in serum. Clin Chem 1984;30:459–61.
20. Jenab M, Bingham S, Ferrari P, et al. Long-term cryoconservation and stability of vitamin C in serum samples of the European prospective investigation into cancer and nutrition. Cancer Epidemiol Biomarkers Prev 2005;14:1837–40.
21. Su SC, Garbers S, Rieper TD, Toniolo P. Temperature variations in upright mechanical freezers. Cancer Epidemiol Biomarkers Prev 1996;5:139–40.
22. Langley MS, Hammond GL, Bardsley A, Sellwood RA, Anderson DC. Serum steroid binding proteins and the bioavailability of estradiol in relation to breast diseases. J Natl Cancer Inst 1985;75:823–9.
23. Phillips GB, Yano K, Stemmermann GN. Serum sex hormone levels and myocardial infarction in the Honolulu Heart Program. Pitfalls in prospective studies on sex hormones. J Clin Epidemiol 1988;41:1151–6.
24. Ocke MC, Schrijver J, Obermann-de Boer GL, Bloemberg BP, Haenen GR, Kromhout D. Stability of blood (pro)vitamins during four years of storage at −20 degrees C: consequences for epidemiologic research. J Clin Epidemiol 1995;48:1077–85.
25. Nomura AM, Stemmermann GN, Heilbrun LK, Salkeld RM, Vuilleumier JP. Serum vitamin levels and the risk of cancer of specific sites in men of Japanese ancestry in Hawaii. Cancer Res 1985;45:2369–72.

Collection, Processing, and Storage of Biological Samples in Epidemiologic Studies: Sex Hormones, Carotenoids, Inflammatory Markers, and Proteomics as Examples

Shelley S. Tworoger and Susan E. Hankinson

Cancer Epidemiol Biomarkers Prev 2006;15:1578-1581.