prokaryotically expressed wild-type p53, thus introducing a bias. A possible explanation of the low classification performance is the analytical specificity, i.e., the ability of an assay to produce a measurable response only for the analyte of interest (20). One weakness of tests A and C is that negative values are obtained at various anti-p53 antibody concentrations. Thus, a higher signal in the well without p53 protein and/or a lower signal in the well with p53 protein gives a final negative result. Considering this, the lack of specific binding to the p53 protein seems to be responsible for the low diagnostic accuracy of tests A and C. Indeed, the specificity of the entire assay depends on the specific binding of the analyte, and thus the anti-p53 antibody, in the environment in which the analytical reaction takes place (21).

One limitation of our results was the control group that was selected. Ideally, the control population should consist of age-matched individuals presenting clinical suspicion of malignancy but whose histopathology remained negative for cancer. Because anti-p53 antibodies were reported to be detectable in the preneoplastic stages of lung cancer (15), young healthy blood donors were selected as a control population because such individuals have a low probability to harbor silent cancer. Accordingly, among the three ELISAs assessed, the anti-p53 ELISA (Dianova) showed the highest discrimination ability between young healthy individuals and patients (ages, 66 to 75 years) with various cancer types. However, these findings do not imply that the anti-p53 ELISA (Dianova) will also be useful in differentiating cancer patients from patients with other diseases that have similar symptomatology.

References

Stability of Blood Homocysteine and Other Thiols: EDTA or Acidic Citrate? Jean-Frédéric Salazar,1 Bernard Herbeth,2 Gérard Siest,1,2 and Pierre Leroy1* (1 Centre du Médicament, Faculté des Sciences Pharmaceutiques et Biologiques, B.P. 403-54001 Nancy Cedex, France; 2 Centre de Médecine Préventive, UPRES, B.P. 7-54501 Vandoeuvre-lès-Nancy Cedex, France; * author for correspondence: fax 33-(0)3-83-32-13-22, e-mail pierre.leroy@pharma.u-nancy.fr)

Homocysteine (Hcy), a thiol-containing amino acid resulting from demethylation of methionine (Met), is relevant to the risk of vascular diseases (1). In plasma, total homocysteine (tHcy) includes the free reduced and oxidized forms as well as the Hcy bound by disulfide bonds in proteins. tHcy is frequently increased in patients with coronary, cerebrovascular, or peripheral arterial diseases; the association is independent of most other risk factors for atherosclerosis (2). The simultaneous measurement of other thiols is of interest because most of them are metabolically related and disturbances of their concentrations can correspond to disorders of metabolism. Hcy may either be catabolized to cysteine (Cys) or remethylated to Met (3). In addition, Cys and γ-glutamylcysteine are precursors to glutathione (GSH), and cysteinylglycine (CysGly) is a breakdown product of GSH; this latter plays a major role in defense against oxidative and free radical-mediated cell injury, and its measurement permits the evaluation of oxidative status of cells and tissues (4).

It should be expected that less rigorous blood sampling and treatment conditions are needed than those involved for thiol redox status evaluation (5). For plasma, storage conditions have little influence on tHcy values: tHcy in plasma is stable at −20 °C for at least 3 months and after nine freeze/thaw cycles (6). In contrast, in whole blood, an increase of tHcy is observed after collection because of ongoing metabolism and time-dependent release from erythrocytes (7). The artificial increase in plasma tHcy
occurs at a rate of 1 \( \mu \text{mol/L} \) per hour at room temperature (6, 7). In most studies, blood is drawn in tubes containing potassium EDTA, which are put immediately in crushed ice and centrifuged “as soon as possible”, and then the plasma is frozen. These conditions prevent increases in \( \text{tHcy} \) in whole blood after collection, but they are not always practical. An alternative procedure is needed, especially for large epidemiological studies involving different sample collection sites, where processing with crushed ice is difficult. Use of sodium fluoride (with heparin as anticoagulant) has been proposed to inhibit \( \text{Hcy} \) release from blood cells for 2 h at room temperature (8). More recently, Willems et al. (9) have claimed that blood collection in acidic citrate tubes stabilizes \( \text{tHcy} \) in whole blood; \( \text{tHcy} \) did not increase markedly for 6 h at room temperature after collection, but an increase of 1.3 \( \mu \text{mol/L} \) was found at baseline in \( \text{tHcy} \) measured in samples collected into acidic citrate compared with EDTA samples. \( \text{tHcy} \) has been reported to be stable in blood samples collected into acidic citrate compared with EDTA for \( \text{h} \) delay time periods existing between these two steps in our own laboratory practices. Moreover, we investigated the difference between the two blood-collection media on the \( \text{tHcy} \) concentration by use of a commercially available FPIA.

Blood was drawn by venipuncture of the antecubital vein from healthy laboratory coworkers (n = 21); informed consent was obtained, and the research protocol was in agreement with the Helsinki Declaration. Blood was collected in tubes containing either 1.8 g/L K\(_2\)EDTA (Vacutainer\textsuperscript{TM} Tubes; Becton Dickinson) or 0.5 mol/L acidic citrate (Biopool Stabilyte\textsuperscript{TM}). The EDTA-treated blood was immediately put on crushed ice, and the acidic citrate-treated blood was kept at room temperature (i.e., 24 °C in the present case). Two independent studies were performed with HPLC and FPIA, respectively. The HPLC study used blood from seven subjects (three men and four women; ages, 28–52 years). Within 15 min, each blood sample was divided into six equal volumes; these aliquots were centrifuged (1000 \( g \) for 15 min at 4 °C for EDTA-treated samples and at room temperature for acidic citrate-treated samples) at 15 and 30 min and 1, 2, 4, and 6 h after blood collection. The FPIA study was done with blood from 14 subjects (8 men and 6 women; ages, 27–56 years), using the same methodology at 15 min and 2 h. Plasma samples resulting from the two studies were frozen at −80 °C until analysis.

The plasma thiols were measured with a slightly modified HPLC technique (11) including reduction of disulfides with tri-\( n \)-butylphosphine, precolumn derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide, isotropic reversed-phase chromatography, and spectrophotometric detection. The stock solution of each thiol calibrator was prepared at a concentration of 1.0 mmol/L in 10.0 mmol/L HCl containing 1 mmol/L EDTA and kept at −80 °C for a maximum of 2 months. Calibration curves including five points were constructed daily with further dilutions of stock solutions in 9 g/L NaCl containing 4 mmol/L EDTA, at concentrations of 50–300 \( \mu \text{mol Cys/L} \), 10–50 \( \mu \text{mol CysGly/L} \), 2.5–15 \( \mu \text{mol Hcy/L} \), and 1–10 \( \mu \text{mol GSH/L} \). Plasma samples were quickly thawed at 37 °C. A 200-\( \mu \text{L} \) aliquot of plasma or calibrator was transferred into a 1.5-mL polypropylene tube (in crushed ice), to which we added 100 \( \mu \text{L} \) of the internal standard solution [thioglycolic acid at a concentration of 300 \( \mu \text{mol/L} \) and 50 \( \mu \text{L} \) of tri-\( n \)-butylphosphine (50 mL/L) in dimethylformamide. After the sample was mixed for 10 s with a vortex-mixer, a nitrogen stream was introduced into the tube for 10 s before it was capped. The resulting mixture was incubated in the dark at 4 °C for 30 min, and 200 \( \mu \text{L} \) of a trichloroacetic acid solution (100 g/L) was added for protein precipitation. After centrifugation at 1500g for 15 min at 4 °C, 100 \( \mu \text{L} \) of the supernatant was transferred into a 1-mL autosampler vial and mixed with 30 \( \mu \text{L} \) of 0.5 mol/L NaOH, 250 \( \mu \text{L} \) of 0.2 mol/L borate buffer, pH 9.0, and 50 \( \mu \text{L} \) of a 2.3 \( \mu \text{mol/L} \) 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide solution in dimethylformamide. The resulting mixture was incubated at 50 °C for 20 min under gentle stirring, and then the derivatization reaction was stopped by the addition of 50 \( \mu \text{L} \) of 1.0 mol/L HCl and chilling in crushed ice.
A 20-μL aliquot was injected into a guard column (4 × 4 mm i.d.) packed with end-capped LiChrospher RP18 (5 μm bead size; Merck Darmstadt) and a short-length analytical column (70 × 4 mm i.d.) packed with Nucleosil 100 C_{18} (5 μm bead size; Macherey Nagel). The sample was eluted with 50 mL/L acetonitrile-950 mL/L phosphate buffer (0.1 mol/L), pH 2.5, at a column temperature of 35 °C and a flow rate of 1.0 mL/min. Excitation and emission wavelengths were set at 385 and 515 nm, respectively.

We used the Abbott IMx® plasma assay for tHcy, as described by Shipchandler and Moore (16). The thiol concentrations from the samples collected in the tubes containing acidic citrate were corrected by the dilution factor caused by the fluid (0.5 mL) initially present in the tube.

The HPLC method was linear for the concentrations indicated above (r > 0.990), and its recoveries were >98%. The within-day imprecision (CV; n = 6) measured with a plasma pool was 2.2% for tCys, 1.7% for tCysGly, 1.9% for tHcy, and 7.2% for tGSH at concentrations of 178.0, 27.8, 7.9, and 3.4 μmol/L, respectively. For the IMx assay of tHcy, the intraday CV (n = 6) was 1.0% at a concentration of 12.5 μmol/L. ANOVA with repeated measurements was used to test the significance of differences between thiol concentrations.

Plasma thiol concentrations measured by HPLC using two different blood collection and storage conditions (EDTA at 0 °C and acidic citrate at room temperature) for delay periods before centrifugation of 15 min to 6 h are shown in Fig. 1.

Differences of thiol concentrations noted between the two collection media were as follows: whatever the delay before centrifugation, tCys concentrations were higher in EDTA plasma than in acidic citrate plasma (6.1% at baseline and 5.3% at 6 h), whereas tGSH concentration were lower (−10.9% at baseline and −8.3% at 6 h), all tests being statistically significant (P < 0.05). tHcy concentrations were higher in EDTA than in acidic citrate plasma (5.2% at baseline and 4.6% at 6 h). A previous report (9) found that tHcy was significantly lower in EDTA than in acidic citrate medium at baseline and at any delay period (−9.3% at baseline and −10% at 6 h) in the same conditions of temperature and storage time as in the present work. No difference in tCysGly concentrations was observed.

Over the time points studied, the time to centrifugation after collection of blood in either EDTA or acidic citrate collection tubes did not significantly affect the concentrations for these thiols except for tCysGly, the concentration of which varied slightly with time (0.5 μmol/L per hour in both collection media). We noticed no significant difference in tCysGly concentrations between EDTA and acidic citrate media at the delay time of 2 h, as reported previously (7); this fact could be also related to the changes of tCys observed between EDTA and acidic citrate media. A hypothetical mechanism could be a leak of GSH from erythrocytes, in which its concentration is much higher than in plasma [3.6 μmol/L in plasma and 0.9 mmol/L in whole blood, respectively (15)], followed by a degradation pathway via reactions catalyzed by γ-glutamyltranspeptidase and dipeptidases (EC 3.4.13.6). The tGSH concentration in plasma is also lower in EDTA than in acidic citrate medium, probably because the same enzymatic reactions are involved. As a matter of fact, a simultaneous decrease of GSH and increase of Cys and CysGly in plasma have been observed as a function of blood storage period between collection and centrifugation (5, 13), and a specific γ-glutamyltranspeptidase inhibitor such as l-serine-borate added to the collection medium (14, 15, 17, 18) seems to be a good alternative to keep the concentration of these thiols stable as a function of time.

The results of the FPIA assays are shown in Table 1. During the restricted delay period (2 h) corresponding to a more practical approach in our laboratory, a slight but
significant increase as a function of time was observed (4.9% between 15 min and 2 h in both media). The difference in tHcy measurement by FPIA between the two collection media was not significant (P > 0.05). Such an absence of difference was opposite to data obtained with HPLC in the present study and in a previous study (9). It could be explained by less selectivity of FPIA than HPLC methods.

In conclusion, tCys, tHcy, and tGSH concentrations measured with HPLC remain stable for 6 h in whole blood, whatever the anticoagulant used: EDTA at 0 °C or acidic citrate at room temperature. This latter approach is a good alternative when the use of EDTA medium collection is not possible, in particular in epidemiological surveys involving several centers. However, because of the significant differences between the mean concentrations in these two media, reference values need to be established that take into account the anticoagulant used. For CysGly, a different strategy must be defined to keep its concentration stable over the same blood storage period. The slight increase in tHcy as a function of time in both collection media that we observed only when using FPIA cannot be fully explained at the present time. The prime priority is thus to define optimal collection and treatment conditions and to keep them constant in an overall study.

We are indebted to the preclinical staff and laboratory department of the Centre de Médecine Préventive (Vandoeuvre-lès-Nancy, France) and to the participating subjects who made this study possible. We particularly wish to thank Dominique Aguillon, Maryvonne Chaussard, Chantal Lafaurie, and Marie-Pierre Recouvreur. We also thank Abbott Laboratories (Abbott Park, IL) for providing FPIA IMx reagents free of charge.

Table 1. Plasma concentrations of tHcy measured by FPIA as a function of blood anticoagulant and delay times between collection and centrifugation.

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>15 min</th>
<th>2 h</th>
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<tbody>
<tr>
<td>EDTA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.2 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.6 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acidic citrate</td>
<td>9.4 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.9 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>Mean ± SD; n = 14.
<sup>b</sup>EDTA at 0 °C; acidic citrate at room temperature.
<sup>c</sup>Significant medium and time effects (ANOVA for repeated measurements, statistical significance accepted at P < 0.05).

Active Ceruloplasmin in Cervicovaginal Secretions: Its Association with Term Premature Rupture of the Membranes, Mitsuharu Ogino,<sup>1,2</sup> Shuichi Hiyamuta,<sup>3</sup> Akihiko Kadota,<sup>3</sup> Yuko Io,<sup>4</sup> and Makoto Hanazono<sup>5</sup> (1 Department of Obstetrics and Gynecology and 2 The Clinical Research Center, Teikyo University School of Medicine, Ichihara Hospital, 3426-3 Anesaki, Ichihara, Chiba 299-0111, Japan; 3 Central Research Laboratories, IDEMITSU KOSAN Co., Ltd., 1280 Kami-izumi, Sodegaura, Chiba 299-0293, Japan; 4 Inoue Ladies’ Clinics, 1-26-9 Fujimi-chou, Tachikawa, Tokyo 190-0013, Japan; <sup>5</sup> author for correspondence: fax 81-436-61-4773 or 81-3-3314-2443, e-mail mogino@cb3.so-net.ne.jp)

Cervicovaginal secretions (CVSs) contain cytokines of maternal origin (1, 2), inflammatory cells, and proteolytic enzymes derived from leukocytes (3, 4) and act to protect the intraamniotic compartment from infectious agents that may ascend along the birth canal. CVSs have been found to contain ceruloplasmin (Cp) (5), a protein formed predominantly in the liver. The potential functions of Cp have been divided into two categories: (a) transportation of copper to tissue sites, and (b) functions such as oxidase activity of aromatic amines and serum antioxidation. In this setting, Cp acts as an extracellular scavenger of free radicals and superoxide ions and endogenously modulates inflammatory responses (6). The former function uses inactive Cp, and the latter function is carried out by active Cp. Hiyamuta et al. (7), using an original ELISA method that they established, found both inactive and...