Interference of Blood Cell Lysis on Routine Coagulation Testing

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Context.—Preanalytical factors influencing the reliability of laboratory testing are commonplace. It is traditionally accepted that hemolytic samples are unsuitable for coagulation assays because of the release of hemoglobin, intracellular components, and thromboplastic substances from damaged blood cells.

Objective.—To evaluate the interference of blood cell lysis on routine coagulation testing.

Design.—Twelve aliquots prepared by serial dilutions of homologous lysed samples collected from 10 different subjects, and displaying a final percentage of lysis ranging from 0% to 9.1%, were tested for prothrombin time, activated partial thromboplastin time, fibrinogen, and dimerized plasmin fragment D. Lysis was achieved by subjecting citrated whole blood to a freeze-thaw cycle.

Outcome Measures.—Interference from blood cell lysis on routine coagulation testing.

Results.—Statistically significant increases in prothrombin time and dimerized plasmin fragment D were observed in samples containing final lysate concentrations of 0.5% and 2.7% respectively, whereas significant decreases were observed in activated partial thromboplastin time and fibrinogen in samples containing a final lystate concentration of 0.9%. The current analytical quality specifications for desirable bias are ±2.0% for prothrombin time, ±2.3% for activated partial thromboplastin time, and ±4.8% for fibrinogen. Percent variations from the baseline values exceeding the current analytical quality specifications for desirable bias were achieved for lystate concentrations of 0.9% (prothrombin time and activated partial thromboplastin time) and 1.8% (fibrinogen), corresponding to average free plasma hemoglobin concentrations of 1.7 and 3.4 g/L, respectively.

Conclusion.—Our results confirm that, although slightly hemolyzed specimens might still be analyzable, a moderate blood cell lysis, as low as 0.9%, influences the reliability of routine coagulation testing. Because the interference in coagulation assays has a wide interindividual bias, we do not recommend lysis correction and we suggest that the most appropriate corrective measure should be free hemoglobin quantification and sample recollection.

Arch Pathol Lab Med. 2006;130:181-184

Coagulation testing is a central aspect of the diagnostic approach to patients with hemostasis disturbances, and it is pivotal for monitoring antithrombotic therapies with either heparins or oral anticoaguilants. Among major determinants of coagulation testing, the standardization of the preanalytical phase exerts a major influence on result reliability. A standardized procedure for specimen collection was demonstrated to be essential to achieve accurate and precise measurements, which might finally provide appropriate and suitable clinical information. However, there are additional circumstances besides specimen collection that might influence the results of coagulation testing; these additional circumstances might also generate misleading results and induce an inappropriate diagnostic or therapeutic approach to the patients. Problems arising from a cumbersome blood draw, such as unsatisfactory attempts to draw blood, difficulty locating easy venous accesses, and missing the vein, are anecdotally known to produce major interference in routine coagulation assays, and the use of hemolyzed specimens has been discouraged to avoid unreliable results. However, to our knowledge, little is known about the true influence of unsuitable samples caused by blood cell lysis on routine coagulation testing. Therefore, we evaluated the interference of in vitro blood cell lysis on prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and dimerized plasmin fragment D (D-dimer) testing.

MATERIALS AND METHODS

Experiment Design and Blood Sampling

On the morning of the first day of the evaluation, 4.5 mL of blood were separately collected in 2 siliconized vacuum tubes containing 0.5 mL of 0.105M buffered trisodium citrate (Becton Dickinson, Oxford, United Kingdom), using a 20-gauge, 0.80 × 19-mm Venoject multisample straight needle (Terumo Europe NV, Leuven, Belgium) from 10 healthy volunteers. Volunteers gave an explicit informed consent for the investigation. Volunteers were selected on the basis of a homogeneous range of values for white blood cell counts (3.25–4.98 × 10^9 cells/μL), platelet count (153–281 × 10^3 cells/μL), and hemoglobin concentration (137–146 g/L). The first specimen (sample 1) was gently mixed by inverting the tube 4 to 6 times and immediately stored at −70°C,
whereas the second specimen (sample 2) was gently mixed by inverting the tube 4 to 6 times and then centrifuged at 3000g for 10 minutes at 10°C. Plasma was separated and stored at −70°C.

On the morning of the second day of the evaluation, blood was collected into 6 additional siliconized vacuum tubes containing 0.105M buffered trisodium citrate (Becton Dickinson) using a 20-gauge, 0.9 x 19-mm straight needle. Aliquots from each of the 10 volunteers. All samples were gently mixed by inverting the tube 4 to 6 times, pooled, and divided into 12 aliquots of 2 mL each. Samples 1 and 2 were thawed. Twelve serial dilutions, obtained by mixing samples 1 and 2, were prepared by adding 200 µL of each dilution to the 2-mL aliquots of blood collected on the second day. Final whole blood lysate concentrations in the mixtures of samples ranged from 0% to 9.1%, roughly corresponding to average free plasma hemoglobin concentrations ranging from 0 to 17 g/L, thus, almost representative of the degree of hemolysis that we observe in specimens sent to our laboratory. The blood samples were centrifuged at 3000g for 10 minutes at 10°C, and plasma was separated and immediately analyzed.

**Laboratory Testing**

Hemolysis was assayed by measuring the concentration of free plasma hemoglobin by the reference cyanmethemoglobin method on a UV-1700 Spectrophotometer (Shimadzu Italia Srl, Milan, Italy).4 White blood cell and platelet counts were performed on an ADVIA 120 (Bayer Diagnostics, Newbury, Berkshire, United Kingdom). Routine coagulation measurements were performed on a Behring Coagulation System (Dade-Behring, Marburg, Germany), using proprietary reagents: Thromborel S (lyophilized human placental thromboplastin), Pathromtin SL (vegetable thromboplastin with micronized silica), and Multifibrin U, for PT, aPTT, and fibrinogen testing, respectively. Because hemolytic samples have a greater than usual inherent spectrophotometric absorbance in conventional clotting assays, PT, aPTT, and fibrinogen measurements were performed at a different wavelength (570 nm), uniquely available on the BCS analyzer. Using this alternative assay configuration, the absorbance threshold for recording the coagulation time is dynamically increased by the evaluation software of the analyzer. The threshold level depends on the inherent absorbance and is automatically calculated by the analytic system. Plasma D-dimer was measured with the Vidas DD, a rapid, quantitative, automated enzyme-linked immunosorbent assay with fluorescent detection, on the Mini Vidas Immunoanalyzer (bioMérieux, Marcy l’Etoile, France). Actual reference ranges were between 10.8 and 13.1 seconds for PT, 26.2 and 36.0 seconds for aPTT, 150 and 400 mg/dL for fibrinogen, and less than 500 ng/mL for D-dimer. All measurements were performed in duplicate within a single analytical session and the results were averaged. Analytical imprecision, expressed in terms of the mean interassay coefficient of variation, was between 2% and 5%, according to the manufacturers.

**Statistical Evaluation**

Differences between coagulation measurements on aliquots of the same sample containing serial concentrations of homologous lysate were evaluated by paired Student t test. Statistical significance was set at P < .05. Percentage variations from the baseline value were further compared with the current analytical quality specifications for desirable bias, as derived from the intra-individual and inter-individual variations (±2.0% and ±0.24 seconds for PT; ±2.3% and ±0.69 seconds for aPTT, ±4.8% and ±15 mg/dL for fibrinogen, respectively).1 This comparison was not feasible for D-dimer testing, because no definitive data on its biologic variability in healthy individuals are available, to our knowledge.

**RESULTS**

Results of our investigation are synthesized in the Figure. The addition of blood cell lysates generated a consistent and dose-dependent trend toward overestimation of PT (Figure, A) and D-dimer values (Figure, D), whereas aPTT (Figure, B) and fibrinogen (Figure, C) values were substantially decreased when compared with the baseline specimens (no lysate). Statistically significant differences by the Student paired t test were observed in samples containing a final lysate concentration of 0.5% for PT, 0.9% for aPTT and fibrinogen, and 2.7% for D-dimer. Percent variations from the baseline value exceeding the current analytical quality specifications for desirable bias were achieved for lysate concentrations of 0.9% (PT and aPTT) and 1.8% (fibrinogen), corresponding to average free plasma hemoglobin concentrations of 1.7 and 3.4 g/L, respectively. There was a roughly linear relationship between the degree of variation and the percentage of lysate in the plasma. However, an unpredictable, sample-specific response was observed for each of the parameters assayed, as shown by the amplitude of the mean coefficients of variation, which ranged from 24% to 28% for PT, from 19% to 20% for aPTT, from 23% to 27% for fibrinogen, and from 54% to 62% for D-dimer. Because the clotting assays were performed at the alternative 570-nm wavelength, these variations were almost referable to the direct effect of hemoglobin, intracellular components, and thromboplastic substances released by damaged cells, rather than to an optical interference.

**COMMENT**

Hemolysis, causing leakage of hemoglobin and other internal components from the erythrocyte membrane into the surrounding fluid, is usually defined for extracellular hemoglobin concentrations greater than 0.3 g/L (18.8 mmol/L). Hemolysis confers a detectable pink-to-red hue to serum or plasma and becomes clearly visible in specimens containing as low as 0.5% hemolytically.8 Hemolyzed specimens are a rather frequent occurrence in laboratory practice, and the relative prevalence is described as being as high as 3.3% of all of the samples afferent to a clinical laboratory.9 Hemolysis, and blood cell lysis in general, are caused by biochemical, immunologic, physical, and chemical mechanisms. In vivo blood cell lysis, which can arise from hereditary, acquired, and iatrogenic conditions (such as autoimmune hemolytic anemia, severe infections, intravascular disseminated coagulation, or transfusion reactions), does not depend on the technique of the healthcare provider; thus, it is virtually unavoidable and not solvable.10 Conversely, in vitro blood cell lysis might be prevented, because it is usually caused by inappropriate specimen collection, handling, and processing. In the case of specimen collection, hemolysis might result from cumbersome or traumatic specimen collection and processing, such as unsatisfactory phlebotomy attempts, difficulty locating venous accesses, prolonged tourniquet time, wet-alcohol transfer from the skin into the blood specimen, small or fragile veins, missing the vein, syringes or butterfly collection devices, small-gauge needles, partial obstruction of catheters, vigorous tube mixing and shaking, or exposure to excessively hot or cold temperatures, although slow leakage may also occur.11 Hemolysis and blood cell lysis may not be evident until centrifugation of the whole blood specimen has been performed, exposing the serum or plasma to scrutiny.

Consistent quality specimens can only result from proper training and the knowledge of the factors that can influence laboratory results. Hemolysis, reflecting a more generalized blood cell lysis, is the most frequent reason for specimen rejection, as indicated by the College of
Influence of blood cell lysis on prothrombin time (PT; A), activated partial thromboplastin time (aPTT; B), fibrinogen testing (C), and dimerized plasmin fragment D testing (D-dimer; D). Differences are given in percentage (mean ± SD) from the baseline sample (no lysis). The dashed horizontal lines indicate the current limits of the analytical quality specifications for desirable bias. Statistically significant differences are evaluated by Student paired t test († P < .05; ‡ P < .01).

American Pathologists Chemistry Specimen Acceptance Q-Probes study. In fact, the release of hemoglobin and additional intracellular contents from erythrocytes, white blood cells, and platelets into the surrounding fluid might falsely elevate measurable levels of the same substances in serum and plasma, or might cause dilution effects, which may compromise the reliability of laboratory testing. Additionally, plasma hemoglobin might increase the optical absorbance or change the blank value, producing method-dependent and analyte concentration-dependent spectrophotometric interference with common laboratory assays. It is traditionally accepted that both in vivo and, more commonly, in vitro blood cell lysis can cause preanalytical variability. Although the amount of interference will depend on the degree of lysis and on the specificity of the method being used, several laboratory results can be affected, especially potassium, sodium, calcium, magnesium, bilirubin, haptoglobin, total protein, aldolase, amylase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, phosphorus, alkaline phosphatase, acid phosphatase, γ-glutamyl transpeptidase, folate, and iron measurements. It is traditionally known that routine coagulation testing might also be influenced by blood cell lysis. The interference is not necessarily only caused by hemoglobin, because many substances are released from blood cells and could influence coagulation assays. In fact, it is more likely that the effects we observed are caused by release of intracellular and thromboplastic substances from either leukocytes or platelets, which are thought to be responsible for shortening the aPTT, as reported by Garton and Larsen. However, there is no definitive evidence or agreement in the current literature on this topic, to our knowledge. Among laboratory testing, PT, aPTT, fibrinogen, and D-dimer measurements are thought to be more susceptible to variations in the preanalytical phase. Results of our investigation indicate that a slight lysis, as low as 0.9%, might influence the reliability of some coagulation testing. We are aware that our investigation has 2 major limitations. First, we reproduced in vitro blood cell lysis by freezing blood specimens at −70°C for up to 24 hours. In addition to lysed erythrocytes, hemolyzed specimens encountered in the laboratory practice usually contain destroyed or fragmented platelets and leukocytes. Therefore, our method seems to be a suitable surrogate, although it is not representative of all of the possible events that can induce whole blood lysis in laboratory practice, especially those represented by troublesome blood collection. Second, we evaluated interference on testing of single reagents and with defined analyzers; therefore, our results might not be universally reproducible or transferable to other testing systems. This is particularly true for analyzers that measure the extent of hemolysis by comparing the absorption of samples at 2 wavelengths, or allow performance of coagulation assays at alternative wavelengths, such as 570 nm, for turbid, icteric, and hemolytic samples. The light source of the Beh-
Ring Coagulation System photometer is a xenon flasher lamp with broadband emission; an interference filter with an appropriate main wavelength is swung into the beam of the light source to obtain light with the desired wavelength. The instrument does not identify hemolytic specimens with a specific flag. However, in our experience, results obtained by conventional assays were systematically flagged as questionable by the addition of a question mark, starting from a final percentage of lysis ranging from 0.5% to 0.9%.

The issue of hemolysis and blood cell lysis has plagued clinical laboratories and continues to be a growing concern. The most frequent causes, such as sampling errors, are avoided by using standardized materials and methods for the preanalytical processes and by training and individual counseling. Although hemolyzed and unsuitable samples are unlikely to be observed in a problem-free phlebotomy activity, each laboratory should document the procedures that are influenced by blood cell lysis and to what extent they are affected. Some instruments report an ability to correct the results for hemolysis, but after our experience, and, at variance with other observations, we do not recommend hemolysis correction in clinical practice, given the wide interindividual and analytical variations arising from the interference. Therefore, if hemolysis and blood cell lysis results from an in vitro cause, we suggest that the most convenient corrective measure might be free hemoglobin quantification and sample recollection.

References