Interference in Coagulation Testing: Focus on Spurious Hemolysis, Icterus, and Lipemia

Giuseppe Lippi, MD[1] Mario Plebani, MD[2] Emmanuel J. Favaloro, PhD, FFSc (RCPA)[3]

[1] Dipartimento di Patologia e Medicina di Laboratorio, U.O. Diagnostica Ematochimica, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy
[2] Dipartimento di Medicina di Laboratorio, Azienda Ospedaliera-Università di Padova, Padova, Italy
[3] Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, Westmead, Australia

Address for correspondence Giuseppe Lippi, MD, Dipartimento di Patologia e Medicina di Laboratorio, U.O. Diagnostica Ematochimica, Azienda Ospedaliero-Universitaria di Parma, Strada Abbeveratoia 2/a, 43100, Parma, Italy (e-mail: glippi@ao.pr.it; ulippi@tin.it).

Semin Thromb Hemost 2013;39:258–266.

Abstract

The chance that errors might jeopardize the quality of testing is inherently present throughout the total testing process, especially in the preanalytical phase. In the coagulation laboratory, as well as in other areas of diagnostic testing, spurious hemolysis, icteria, and lipemia in test samples represent by far the leading diagnostic challenges. Interference in hemostasis testing due to spurious hemolysis is attributed to both analytical and biologic elements, namely high absorbance of cell-free hemoglobin at wavelengths used by optical instrumentation and release of both cytoplasmatic and plasma membrane molecules (e.g., tissue factor, proteases, phospholipids, and ADP) that can spuriously activate blood coagulation and platelets. The interference attributable to hyperbilirubinemia is mostly due to spectral overlap, whereas that of hypertriglyceridemia mainly reflects elements of light scatter and volume displacement as well as direct interference of lipid particles with hemostasis. In practical terms, spurious hemolysis reflects a more generalized process of endothelial and blood cell damage, so that test results on spuriously hemolyzed specimens should be systematically suppressed. The bias attributable to hyperbilirubinemia is less significant using modern coagulometers equipped with dedicated wavelengths (i.e., with readings at 650 nm or above), so that test results in samples with a bilirubin concentration up to 20 mg/dL can still be analytically reliable. The interference observed in lipemic samples is most evident with readings using wavelengths lower than 500 nm and can hence be prevented with readings at 650 nm or above, and/or using higher dilutions of the test sample, or can be abated in high hypertriglyceridemic specimens (i.e., > 1,000 mg/dL) using high speed microcentrifugation or lipid extraction with organic solvents such as fluorine-chlorinated hydrocarbon, or lipid-clearing agents such as LipoClear (StatSpin Inc., Norwood, MA) and n-hexane.

Keywords

- interference
- coagulation testing
- hemolysis
- lipemia
- errors

The total testing process develops through a broad series of operations and activities, wherein the analytical phase is only the central portion, being respectively preceded and followed by the preanalytical and postanalytic phases, but all of which contribute to complete the so-called "brain-to-brain loop."[1,2] Throughout this complex series of processes, the chance of errors being made that may ultimately jeopardize the quality of testing, patient safety, or both is inherently high, especially in the presence of a poor degree of standardization and when a hierarchical quality management system is not thoughtfully

published online December 10, 2012

Issue Theme Quality in Hemostasis and Thrombosis, Part II; Guest Editors, Giuseppe Lippi, MD, Mario Plebani, MD, and Emmanuel J. Favaloro, PhD, FFSc (RCPA).

Copyright © 2013 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662.

DOI http://dx.doi.org/10.1055/s-0032-1328972.
ISSN 0094-6176.

Downloaded by: Universita di Parma. Copyrighted material.

applied.[3–5] Several lines of historical as well as contemporary evidence support the hypothesis that processes comprised within the preanalytical phase are the most vulnerable to errors and thus most easily flaw-prone, due to inappropriate standardization, poor adherence to best practice, and involuntary human mistakes.[6–9] In the coagulation laboratory, spurious hemolysis is by far the leading source of interference in plasma samples (40%), prevailing over pre-analysis clotting (29%), inappropriate filling of primary blood collection tubes (28%), contamination from infusion route (2%), lipemia, and hyperbilirubinemia (both approximately 1%),[10] whereas lipemia (13%) and hyperbilirubinemia (11%) both precede spurious hemolysis (4%) as prevailing nonconformances in whole blood specimens.[11] It is, however, noteworthy that the inherent complexity of hemostasis and the sophistication of analytical methods and instrumentations make the coagulation laboratory more vulnerable to preanalytical errors and interferences than other areas of in vitro diagnostics.[12] This is also clearly recognized in the most recent H21-A5 standard of the Clinical and Laboratory Standards Institute (CLSI),[13] which advises that samples that are hemolyzed, icteric, lipemic, or that contain substances that potentially interfere with light transmission represent a serious challenge for hemostasis testing.

Spurious Hemolysis

Hemolyzed specimens are the leading source of nonconformance in several areas of diagnostic testing,[14,15] including clinical chemistry,[16] immunochemistry,[17] complete blood cell counting,[18] arterial blood gas analysis,[19] and—last but not least—platelet function[20,21] and coagulation testing.[12,22] Once the likelihood of in vivo hemolysis (i.e., hemolytic anemia) has been ruled out, these samples pose the most serious problem for the laboratory due to the potential for both analytical and biologic interference. The former is mostly attributable to the strong absorbance of cell-free hemoglobin still present in the sample after centrifugation at those wavelengths that are conventionally used by the (optical) instrumentation for coagulation testing (►**Fig. 1**). Thus, high baseline absorbance values are detected by the instrument, and test results will either be unreliable or not reported. The latter interference is often overlooked but is probably just as important because it affects both optical and mechanical instrumentation and can be attributed to release of both cytoplasmatic and plasma membrane molecules (e.g., tissue factor, proteases, phospholipids, and adenosine diphosphate [ADP], among others), which can produce spurious activation of blood coagulation and platelets, generation of—seldom insignificant—blood clots, which may further bias test results for premature activation of blood coagulation (i.e., shortening of clotting times) or consumption of clotting factors (i.e., prolongation of clotting times), and hyperactivation of platelets. Moreover, the presence of cell-free hemoglobin frequently reflects problems related to the collection of blood specimens (e.g., a cumbersome venipuncture), so that it can be considered a reliable index of concomitant endothelium injury with release of thromboplastin-like procoagulant activity and exposure of the subendothelial surface, which would ultimately compromise the quality of the specimen (►**Fig. 2**). In any of these cases, test results should be suppressed and not reported to the requesting clinician. This would be true for both optical and mechanical detection systems because the biologic effects are significant, even if the analytical effects on mechanical systems are negligible.

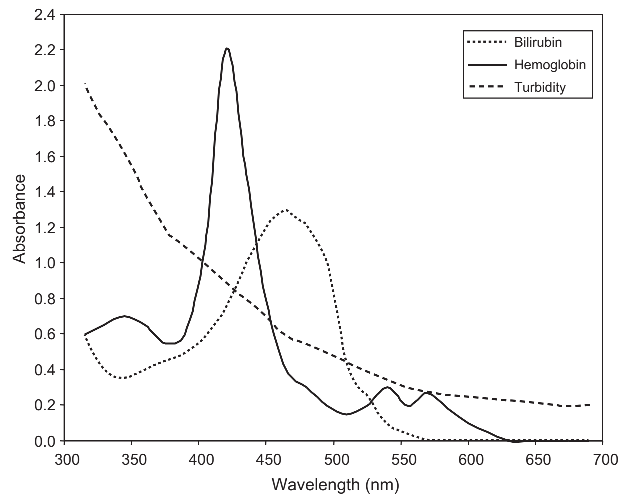

Fig. 1 Absorbance of potentially interfering substances (i.e., cell-free hemoglobin, hyperbilirubinemia and lipemia [turbidity]).

The chance of producing hemolyzed samples spans throughout the preanalytical phase and thereby comprises sample collection,[23] handling,[24] and transport as well as preparation or processing of samples for testing[25] (►**Table 1**). The problem may be largely amplified in organizational settings where preanalytical processing modules are physically attached to analytical platforms, and these "hide" specimens from visual inspection, from the point of receipt in the laboratory until the analyses have been completed.[26] Technically speaking, hemolyzed plasma can be classified according to the concentration of cell-free hemoglobin, that is, nonhemolyzed ($\leq$ 0.05 g/L, yellow hue), slightly hemolyzed (from 0.05 to $\leq$ 0.3 g/L, yellow to slightly pink hue), mildly hemolyzed (0.3 to $\leq$ 0.6 g/L, pink to slightly red hue), frankly hemolyzed ($\leq$ 0.6 to 2.0 g/L, slightly red hue), and grossly hemolyzed ($\geq$ 2.0 g/L, red to brown hue).[27] In daily practice, the vast majority of hemolyzed specimens—approximating 95%—are slightly hemolyzed (i.e., cell-free hemoglobin $\leq$ 0.3 mg/L)[28] and are most prevalently referred from the emergency department and intensive care units.[29] Hemolysis is most frequently identified by the laboratory personnel through visual scrutiny, once the sample has been centrifuged and when cell-free hemoglobin in serum or plasma is greater than 0.3 g/L, which reflects a lysis of 2.0 to 2.3% of red blood cells in anticoagulated blood samples with total hemoglobin concentration comprised between 13.0 and 15.0 g/L. The serum indices (which also include the hemolysis index) are now widespread in preanalytical modules and clinical chemistry platforms,[30] whereas they have only recently been implemented in coagulation instrumentation (e.g., the so-called "HIL-System" on CS-2100i and CS-5100, Sysmex

Downloaded by: Universita di Parma. Copyrighted material.

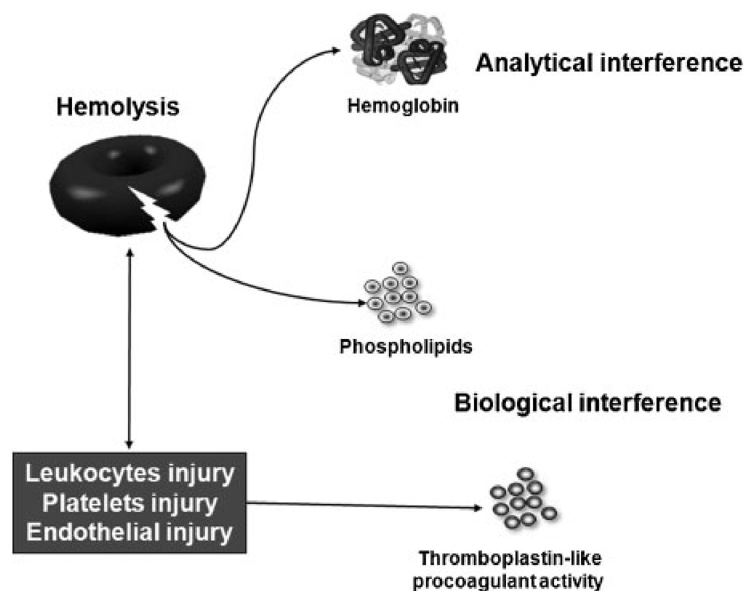

Fig. 2 Leading mechanisms causing hemolysis-related interference in coagulation testing.

Company, Kobe, Japan).[31,32] A more consolidated feature in modern coagulometers is, however, the possibility to use multi-wavelength detection (e.g., simultaneous analysis at 340, 405, 575, 660, and 800 nm), which would enable selection of the appropriate absorbance for specific types of interfering substances such as lipemia and bilirubin other than hemoglobin, thus allowing practitioners to process those samples with a modest degree of photometrical interference.

Technical Considerations on Hemolysis Studies

One of the leading problems in finding consistency across the different studies it the vast array of techniques used to test hemolysis interference. Basically, these include spiking plasma with hemolysate preparations, freezing and thawing whole anticoagulated blood, lysis of whole anticoagulated samples by means of deionized water with or without detergents, mechanical lysis of whole anticoagulated blood

Table 1 Leading causes of sample hemolysis

1. Sample collection
a. Poor venous access
b. Inappropriate technique
c. Traumatic venipuncture
d. Use of syringes rather than vacuum system
e. Use of needle of smaller size (i.e., lower than 23 gauge)
f. Underfilling of primary blood tubes
g. Prolonged tourniquet placing
h. Fist clenching
2. Sample handling
a. No mixing or excessive shaking of collection tubes
3. Sample transportation
a. Excessive time before centrifugation
b. Transport at extremes of temperature (too low or too high)
c. Trauma and mechanical injury to the specimens
4. Sample preparation
a. Centrifugation at inappropriate force (i.e., too high), time (i.e., too long) and/or temperature (i.e., too low or too high)
b. Generation of a poor barrier separation or re-spun sample
c. Transfer of plasma containing contaminating cell fractions, followed by freezing and thawing before testing

Downloaded by: Universita di Parma. Copyrighted material.

by sonication, stirring with a metallic bar, and application of the blade of a tissue homogenizer and aspiration through a fine blood collection needle (i.e., lower than 25 gauge).[33] Although each of these approaches carries its own advantages and limitations, the most reliable techniques seem to be those that more closely reproduce mechanical hemolysis of blood during collection, which is incidentally the leading source of spurious hemolysis throughout the preanalytical phase. As such, spiking samples with hemolysate or pure hemoglobin solution is considered unsuitable because this method would exclude the potential biologic interferences attributable to the lysis of leukocytes and platelets, which may be injured together with the erythrocytes.[18] The method of freezing whole anticoagulated blood appears more suited, but a highly standardized technique is necessary because temperature and duration of freezing are critical, and will otherwise produce a heterogeneous, osmotically induced injury to blood cells.[34] The preferable approach is therefore that based on mechanical injury to whole anticoagulated blood, which would technically mimic the physical damage to the plasma membrane of all types of blood cells that may occur during drawing and handling of the specimen. Among "injury techniques," the one that most closely reproduces the breakdown of cells due to traumatic blood collection is that originally proposed by Dimeski[35] and further modified by Lippi et al[36]; this entails serial aspiration of whole anticoagulated blood by a 0.5 mL insulin syringe equipped with a very thin needle (e.g., 30 gauge or lower). It is also noteworthy that the number of aspirations is directly linked to the degree of damaged blood cells and cell-free hemoglobin, so that an arbitrary scale of hemolysis can be easily and suitably reproduced.

The identification of reliable thresholds of bias is the second critical issue in hemolysis as well as in other interference studies. Different approaches can be used also in this circumstance. Regardless of appreciation of an analytical bias in unsuitable samples, which may still be technically meaningful but otherwise clinically negligible in many occasions, the use of thresholds derived from reliable biologic evidence is advisable (e.g., the use of the critical difference of the quality specification criteria based on biologic variability).[37,38] Whenever the percentage of absolute bias exceeds the boundaries of clinical significance, there is evidence that test results should be systematically suppressed.

Another aspect to be considered is the appropriate selection of patient samples for these studies. Although this issue would apply to any type of interference study as well as any type of laboratory parameter, the appropriate selection of the reference samples is more critical in the hemostasis laboratory, wherein specimens with abnormal values (exceeding either the lower or upper limit of the reference range) may display a rather different behavior to samples yielding normal values. This is specifically due to the nature of analyses such as the prothrombin time (PT),[39] activated partial thromboplastin time (APTT),[40] D-dimer,[41] and even platelet function testing,[42] which are considered "global tests" that thereby reflect multiple abnormalities of clotting factors or platelets rather than a punctiform and circumstantiate defect. As such, this type of investigation should not only be performed on both "normal" and "abnormal" plasma samples, but the latter specimens should also be adequately heterogeneous (e.g., representative of clotting factor deficiencies as well as collected from patients undergoing therapy with various antiplatelet[43] or anticoagulant drugs,[44] especially different formulations of heparins,[45] vitamin K antagonists,[46] or the novel oral anticoagulants).[47–49] A final issue is the test methodology, wherein the different combination of reagents and instrumentations makes comparison across different studies very difficult, so that a local evaluation of bias may be advisable.

Influence of Hemolysis on Hemostasis Testing

Only a few studies have been published so far on the influence of spurious hemolysis on coagulation and hemostasis testing and, unfortunately, these are barely comparable due to the use of different techniques for producing the hemolysate, different patient samples, heterogeneous criteria to assess the bias, as well as different instrumentation and reagents. Thus, direct comparison is essentially unfeasible and potentially misleading; however, some general points can be usefully identified.

As regards routine coagulation testing, in 1998, Roß and Paar assessed the interference due to hemolysis using the MDA 180 analyzer (BioMérieux, Marcy l'Etoile, France),[50] and observed that results of APTT were relatively unbiased by spiked hemoglobin, whereas PT exhibited significant deviations (shortening) at concentrations of hemoglobin > 30 µmol/L, thrombin time (shortening) at concentrations of hemoglobin > 210 µmol/L, fibrinogen (decrease) at concentrations of hemoglobin > 60 µmol/L, and antithrombin (decrease) at concentrations of hemoglobin > 150 µmol/L. Laga et al investigated pairs of hemolyzed (cell-free hemoglobin concentration from 0.2 to 7.0 mg/L) and subsequently recollected nonhemolyzed (cell-free hemoglobin concentration < 0.3 mg/L) patient samples for PT, APTT, and some clotting factor assays.[51] Additional nonhemolyzed specimens from healthy volunteers were also subjected to mechanical hemolysis by means of a tissue homogenizer. In hemolyzed patient plasma samples, both PT and APTT measured on an MDA-II (BioMérieux) were significantly shortened (-3.0 and -2.8%, respectively). As regards the clotting factor assays, significant differences were observed for activated factor VII ($+32.6$%), factor V ($+11.9$%), factor X ($+2.6$%), and for prothrombin fragment 1.2 ($+125$%), but not for factor XIIa and factor VIII. Paradoxically, different results were obtained when assessing mechanically hemolyzed plasma specimens, wherein no bias was observed for PT, whereas a prolongation (rather than a shortening) was observed for APTT in hemolyzed specimens (from $+1.0$ to $+8.6$% in samples with 0.9 to 3.3% hemolysis, i.e., from approximately 1.8 to 6.8 g/L cell-free hemoglobin). Interestingly, no correlation was found between the degree of hemolysis and the relative bias (either shortening or prolongation) of both PT and APTT in patient specimens.

We have also prepared aliquots by serial dilutions of homologous samples collected from 10 different health volunteers spiked with hemolysate produced by a single freezing-thawing cycle, to obtain a final percentage of lysis ranging

Downloaded by: Universita di Parma. Copyrighted material.

from 0 to 9.1%, which correspond to a range of cell-free hemoglobin between 0 and 17 g/L.[52] These aliquots were then tested for PT, APTT, fibrinogen on a Behring Coagulation System (Dade-Behring, Marburg, Germany), and D-dimer on Mini Vidas (BioMérieux). Although analytically significant interference was recorded at cell-free hemoglobin concentrations of 0.9 g/L for PT (prolongation), 1.7 g/L for APTT (shortening) and fibrinogen (decrease), and 5.0 g/L for D-dimer (increase), clinically significant variations were observed at 1.7 g/L for PT and APTT (prolongation and shortening, respectively) and 3.4 g/L for fibrinogen (decrease). The value of D-dimer exhibited a bias exceeding 10% only at a cell-free hemoglobin concentration of 13.6 g/L or higher. Likewise clinical chemistry testing,[53] and in agreement with the data of Laga et al,[51] the sample-specific bias was predominantly unpredictable, with coefficient of variations as high as 62%, which virtually prevented the use of any equation to correct test results for hemolysis degree, thus validating that the decision to suppress test results on hemolyzed specimens is not empirical. This is in agreement with the current CLSI standard, recommending that samples with visible interfering substances (including hemolysis) should not be processed for clotting assays due to potential activation of the coagulation cascade and interference with end point measurements.[13]

More recently, Tantanate et al also spiked hemolytic hemoglobin to residual sodium-citrated patient plasma with normal PT, APTT, and fibrinogen values on Sysmex CS-2100i[54] and showed that all tests exhibited a clear trend toward increased values in hemoglobin-spiked specimens. In a subsequent study, we assessed the influence of mechanical hemolysis of anticoagulated blood on two different D-dimer immunoassays, the former based on an immunoturbidimetric technique (HemosIL D-dimer HS for ACL TOP, Instrumentation Laboratory, Bedford, MA) and the latter on a chemiluminescent immunoassay (HemosIL AcuStar D-dimer, Instrumentation Laboratory).[55] It is noteworthy that a rather similar trend toward reduced values was observed with both methods on four different plasma pools, thus suggesting that the interference from hemolysis seems more biologic than analytical. In agreement with the previous investigation, the overall bias observed in samples with gross hemolysis (i.e., concentration of cell-free hemoglobin $>$ 11.5 g/L) was modest, always lower than 10%, thus indicating that test results obtained in the vast majority of hemolyzed specimens might still be reliable for this parameter and thereby could be safely released to the clinicians.

Platelet function tests may be also negatively biased for the presence of hemolysis and/or generalized cell injury involving erythrocytes, platelets, and leukocytes. We have recently assessed whole blood samples collected from healthy volunteers on Platelet Function Analyzer-100 (PFA-100, Siemens Healthcare Diagnostics, Tarrytown, NY) before and after mechanical hemolysis by aspiration through a very fine needle.[21] When compared with the nonhemolyzed aliquot, 44% of results were flagged as "flow obstruction" for both collagen and ADP (CADP) or collagen and epinephrine (CEPI) in samples containing approximately 6.0 g/L of cell-free hemoglobin, whereas the closure times of the remaining samples were dramatically prolonged with both agonists. In aliquots containing cell-free hemoglobin concentrations as high as 12.0 g/L, the results were flagged as "flow obstruction" in 67% of cases for CADP and in all samples for CEPI, whereas the closure times produced with CADP in the remaining samples were also dramatically prolonged. These results clearly attest that the mechanical injury of blood, which causes considerable release of ADP (mostly from erythrocytes), prothrombotic material (mostly from leukocytes), and cellular debris, along with a potential decrease of platelet count in the specimen, might in fact lead to a various degree of hyperactivation of platelets that may variously interfere with testing, up to generation of flow obstruction in the PFA-100 test cartridges. It is thereby essential, in the presence of unexpectedly prolonged results and/or flow obstruction, to include sample hemolysis as a potential cause of spurious test results. Indeed, should blood be left over following testing by the PFA-100 and identification of a flow obstruction, the sample could be centrifuged and the plasma assessed for evident hemolysis.

The effect of spurious hemolysis on canine kaolin-activated thromboelastography has also been recently investigated by Bauer et al.[56] Blood cells collected from healthy dogs were tested before and after freezing or mechanical injury by repetitions of blood expulsion through a 23-gauge needle. As compared with nonhemolyzed specimens, mechanical injury caused a $>$ 50% decrease of reaction time, whereas freezing remarkably increased coagulation time by nearly three times and decreased the angle by more than 50%. The maximum amplitude was also decreased by approximately 20 and 47% in mechanically hemolyzed and frozen samples, respectively. Finally, both types of hemolysis decreased mean platelet component concentration by about 20 and 26% in mechanically hemolyzed and frozen samples, respectively. These results are hence suggestive for activation of both primary and secondary hemostasis in spuriously hemolyzed samples, whereas the presence of hyporeactive platelets could not be excluded as attested by the reduced clot firmness. No other studies have addressed the issue of hemolysis interference in hemostasis testing to the best of our knowledge.

Hyperbilirubinemia

Serum bilirubin, the final result of the catabolism of heme, is a highly hydrophobic compound transported in blood linked with albumin, with $<$ 0.01% circulating in unbound, free form. The main site of bilirubin metabolism is the liver, where the molecule is dissociated from albumin and then conjugated with one or two molecules of glucuronic acid to form bilirubin monoglucuronide and diglucuronide (i.e., conjugated or direct bilirubin). Hyperbilirubinemia is commonly defined as the presence of excess bilirubin in the blood (i.e., bilirubin concentration $>$ 1.5 mg/dL), and can be classified as conjugated or unconjugated, according to the predominant form.[57] The interference in hemostasis testing due to the presence of hyperbilirubinemia is mostly attributable to spectral overlap (i.e., the compound has a high absorbance

Downloaded by: Universita di Parma. Copyrighted material.

between 400 and 520 nm, with absorbance peak at roughly 456 nm; ►Fig. 1), rather than for its biologic properties (e.g., bilirubin can be rapidly oxidized to biliverdin and bilipurpurin and then reacts with all oxidants present in the sample).[58] As such, once the photometric interference has been eliminated, no other sources of bias should be expected in clotting assays.

Influence of Hyperbilirubinemia on Hemostasis Testing

Although several studies have assessed the interference of hyperbilirubinemia on clinical chemistry parameters, with most of these recently reviewed by Dimeski,[59] there is less abundant information on this type of interference on routine and specialized coagulation testing. The first study dealing with this topic was published in 1998 by Roß and Paar, using an optical test system, the MDA 180 analyzer.[50] After spiking plasma samples with unconjugated bilirubin, the authors concluded that APTT results were virtually unaffected, whereas PT (shortening) and thrombin time (prolongation) exhibited significant deviations at concentrations of bilirubin > 14.6 mg/dL (250 µmol/L); moreover, effects were also observed with fibrinogen (increase) at concentrations of bilirubin > 21.9 mg/dL (375 µmol/L) and antithrombin (decrease) at concentrations of bilirubin > 1.5 mg/dL (25 µmol/L). Quehenberger et al compared results of icteric samples (bilirubin concentration between 46 and 925 mmol/L, i.e., between 2.7 and 54.1 mg/dL) obtained on the optical instrument CA-6000 with those of an STA analyzer (Diagnostica Stago, Cedex, France), which is instead based on mechanical detection of the clot.[60] At variance with the results earlier obtained by Roß and Paar, the correlations between the instruments were satisfactory, always higher than 0.97 except for thrombin time (0.641). Another research group tested icteric plasma samples with the 671 nm wavelength on ACL TOP (Instrumentation Laboratory)[61] and Sysmex CA-7000[62] and found that test results of all coagulation, chromogenic, and immunologic assays obtained on these photo-optical clot detection–based instruments were highly comparable to those obtained using a mechanical clot detection–based analyzer (i.e., STA, Diagnostica Stago). Nearly identical results were reported by Dorn-Beineke et al,[63] who tested icteric samples (i.e., concentrations of total bilirubin from 7.4 to 41.5 mg/dL [127 to 710 µmol/L]) on Sysmex CA-6000 and Sysmex CA-7000 and failed to find any significant bias. Analogously, Tantanate et al spiked exogenous bilirubin to patient plasmas with normal PT, APTT, and fibrinogen values[54] and observed no interference using the 660 nm wavelength on Sysmex CS-2100i up to a final concentration of 14.07 mg/dL (241 µmol/L) of free bilirubin, and 14.55 mg/dL (249 µmol/L) of conjugated bilirubin. According to the data of these recent studies, it therefore seems reasonable to conclude that hyperbilirubinemia does not represent a substantial analytical problem for the modern coagulometers, provided that a dedicated wavelength is used for analysis of visually icteric samples.

Lipemia

The potential origin of "nontransparent turbid milky samples" (more simply "turbid samples" or "lipemic samples") is varied. Basically, turbidity is typically caused by the presence of an excess of triglycerides—usually above 500 mg/dL (i.e., 5.65 mmol/L)—which can be attributed to physiologic causes (e.g., postprandial metabolism), para-physiologic causes (e.g., administration of intravenous lipids), as well as other metabolic disorders such as diabetes, chronic alcohol abuse, impaired renal function, thyroid diseases, acute pancreatitis, myeloma, primary biliary cirrhosis, systemic lupus erythematosus, and medication such as protease inhibitors, estrogen, and steroids.[64,65]

The use of "nontransparent turbid milky samples" (more simply "turbid samples") for laboratory testing carries some technical, analytical and clinical problems. The interference mechanism seem basically attributable to light scatter, reflectance, or absorption, thus causing measurement bias in photometric methods, especially at shorter wavelengths (i.e., Rayleigh law; ►Fig. 1);[58] volume displacement by lipids, which is particularly evident with using indirect ion selective electrodes[66]; and direct interference of lipid particles with hemostasis. Several modern coagulometers now use optical detection of the clot along with optical assays for measuring the absorbance in the samples. It is thereby predictable that no reliable baseline readings will be obtained on highly lipemic specimens, in which case test results will either be unreliable or not reported by the instrument; in either case, test results should be suppressed and not reported to the requesting clinician. Understandably, analyzers using mechanical or electromechanical detection are much less influenced by this type of analytical interference.

Influence of Lipemia on Hemostasis Testing

Roß and Paar first assessed the bias in turbid samples using the MDA 180 analyzer,[50] observing that results of PT and APTT were relatively unbiased by very high concentrations of triglycerides (> 965 mg/dL, i.e., 10.9 mmol/L), whereas thrombin time exhibited significant bias (prolongation) at concentrations of bilirubin > 965 mg/dL (10.9 mmol/L), fibrinogen (reduction), and antithrombin (increase) at concentrations of lipids > 487 mg/dL (5.5 mmol/L). In a subsequent study, Arambarri et al simulated the turbidity due to lipemia by addition of increasing quantities of 20% intralipid to patient plasma samples with normal or pathologic coagulation tests and assessed PT and APTT on an ACL-3000 analyzer (Instrumentation Laboratory).[67] In those specimens with hypertriglyceridemia (i.e., triglycerides > 9.79 mmol/L, 866 mg/dL), no test data could be obtained. Nevertheless, test results were instead produced after treatment of plasma with n-hexane, on average 6% lower for PT and 12% higher for APTT, respectively, compared with control. Quehenberger et al compared results of lipemic samples (triglyceride concentration ranging between 2.42 and 18.70 mmol/L, i.e., 214 to 1,655 mg/dL) obtained on the optical instrument Sysmex CA-6000 with those of a mechanical clot detection-based analyzer, the STA.[60] Final correlations of 0.907, 0.988, 0.288, 0.961, and 0.902 were reported for PT, APTT, thrombin time, fibrinogen, and antithrombin, respectively, indicating a major problem only for thrombin time testing. Dorn-Beineke et al assessed lipemic samples (i.e., triglyceride concentrations

Downloaded by: Universita di Parma. Copyrighted material.

between 3.51 mmol/L and 18.5 mmol/L) on Sysmex CA-6000 and Sysmex CA-7000,[63] describing a negative bias as well as invalid results for PT in samples containing triglycerides > 13.5 mmol/L (1,195 mg/dL), and a positive bias as well as invalid results on Clauss fibrinogen in samples with triglycerides exceeding 3.51 mmol/L (317 mg/dL). The results of APTT were substantially unbiased up to a final triglyceride concentration of 18.5 mmo/L (1,637 mg/dL). Bai et al further compared optical and mechanical clot detection instruments for routine coagulation testing in a large volume clinical laboratory (i.e., Sysmex CA-1500 with readings at 660nm vs. Diagnostica Stago STA), and found a high correlation for PT, APTT, and fibrinogen (all greater than 0.98) when assessing turbid samples.[68] No clot was observed for APTT in only 2.4% of turbid samples. In the study of Tantanate et al, who spiked 20% intralipid to patient plasmas with normal PT, APTT, and fibrinogen values,[54] consistent trends toward increased values of PT as well as toward reduction of APTT and fibrinogen were observed with the Sysmex CS-2100i analyzer. In a recent publication, we have also assessed a vast array of coagulation tests including PT, APTT, fibrinogen, protein C, protein S, and antithrombin on ACL TOP in 17 healthy volunteers eating a standardized meal that contained carbohydrates, protein, and lipids for a total caloric intake of 563 Kcal.[69] Samples were drawn before the meal, immediately after, and in the following 1, 2, and 4 hours. The only clinically significant bias was recorded for APTT, 2 hours after the ingestion of the meal.

Conclusions

Regardless of analytical considerations, which, although important, may not necessarily influence clinical practice and patient safety, the type of interference encountered with spurious hemolysis, icterus, and lipemia is substantially different, and thus requires distinctive approaches.

Spurious hemolysis typically reflects a more generalized process of endothelial and blood cell damage, so that any consideration about the potential optical interference attributable to the absorbance of cell-free hemoglobin is ancillary to the presence of a critical biologic bias caused by release of prothrombotic and proaggregant material from the injured cells. Test results of both routine and specialized hemostasis testing should therefore be systematically suppressed in all samples with visible degree of hemolysis (i.e., cell-free hemoglobin > 0.3 to 0.6 g/L). It may also be advisable to routinely assess the hemolysis index in these samples, clearly establish the degree of potential interference, and decide the most suitable actions. Naturally, test results from patients with in vivo hemolysis should instead be reported to avoid repeated collections with similar outcome, albeit with a caveat detailing the limitation of the test result.

The bias attributable to hyperbilirubinemia is almost exclusively analytical, due to spectral overlap. There is probably insignificant clinical bias using modern coagulometer equipped with dedicated wavelengths (i.e., with readings at 650 nm or above) so that test results may be reliable and hence released to requesting clinicians, particularly in samples with a bilirubin concentration lower than 20 mg/dL (342 μmol/L).

The interference observed in lipemic samples is of both optical and biologic origin and develops from a different source but produces a similar negative influence on testing as that previously described for spurious hemolysis. Due to the most evident interference with readings using wavelengths lower than 500 nm (►**Fig. 2**), the optical bias can, however, be easily prevented using dedicated wavelengths (i.e., > 650 nm) and/or using higher dilutions of the sample. Naturally, sample dilutions can only be applied to assays that already use dilutions (e.g., fibrinogen, factor assays, etc) and not to global assays (e.g., PT, APTT). Because the presence of lipid particles can still bias the measurement for biologic interference, the CLSI currently recommends the removal of excess triglycerides by ultracentrifugation for at least 30 minutes at a speed above $40,000 \times g$.[70] Nevertheless, this approach is time consuming, requires dedicated instrumentation (i.e., the ultracentrifuge), and hence is incompatible with the routine daily practice of most clinical laboratories. Moreover, ultracentrifugation may cause precipitation of the large molecular proteins such as fibrinogen or factor VIII/von Willebrand factor complex. An alternative approach entails high-speed microcentrifugation (e.g., double centrifugation at $> 20,000 \times g$ for 15 minutes) or lipid extraction by means of organic solvents such as fluorine chlorinated hydrocarbon or lipid-clearing agents such as LipoClear (StatSpin Inc., Norwood, MA) and n-hexane.[71]

References

1 Plebani M, Lippi G. Closing the brain-to-brain loop in laboratory testing. Clin Chem Lab Med 2011;49(7):1131–1133
2 Plebani M, Favaloro EJ, Lippi G. Patient safety and quality in laboratory and hemostasis testing: a renewed loop? Semin Thromb Hemost 2012;38(6):553–558
3 Lippi G. Governance of preanalytical variability: travelling the right path to the bright side of the moon? Clin Chim Acta 2009;404(1):32–36
4 Lippi G, Plebani M, Simundic AM. Quality in laboratory diagnostics: from theory to practice. Biochem Med (Zagreb) 2010;20(2):126–130
5 Lippi G, Simundic AM, Mattiuzzi C. Overview on patient safety in healthcare and laboratory diagnostics. Biochem Med (Zagreb) 2010;20(2):131–143
6 Lippi G, Guidi GC, Mattiuzzi C, Plebani M. Preanalytical variability: the dark side of the moon in laboratory testing. Clin Chem Lab Med 2006;44(4):358–365
7 Lippi G, Chance JJ, Church S, et al. Preanalytical quality improvement: from dream to reality. Clin Chem Lab Med 2011;49(7):1113–1126
8 Lippi G, Simundic AM. Total quality in laboratory diagnostics. It's time to think outside the box. Biochem Med (Zagreb) 2010;20(1):5–8
9 Simundic AM, Lippi G. Preanalytical phase—a continuous challenge for laboratory professionals. Biochem Med (Zagreb) 2012;22(2):145–149
10 Salvagno GL, Lippi G, Bassi A, Poli G, Guidi GC. Prevalence and type of pre-analytical problems for inpatients samples in coagulation laboratory. J Eval Clin Pract 2008;14(2):351–353
11 Salvagno GL, Lippi G, Gelati M, Guidi GC. Hemolysis, lipaemia and icterus in specimens for arterial blood gas analysis. Clin Biochem 2012;45(4-5):372–373

Downloaded by: Universita di Parma. Copyrighted material.

12 Favaloro EJ, Adcock Funk DM, Lippi G. Pre-analytical variables in coagulation testing associated with diagnostic errors in hemostasis. Lab Medicine 2012;43(2):1–10
13 Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays; Approved Guideline—Fifth Edition. Wayne, PA: Clinical Laboratory Standards Institute; 2008. CLSI document H21–A5
14 Lippi G, Blanckaert N, Bonini P, et al. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med 2008;46(6):764–772
15 Simundic AM, Topic E, Nikolac N, Lippi G. Hemolysis detection and management of hemolysed specimens. Biochem Med (Zagreb) 2010;20(2):154–159
16 Lippi G, Salvagno GL, Montagnana M, Brocco G, Guidi GC. Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med 2006;44(3):311–316
17 Lippi G, Avanzini P, Dipalo M, Aloe R, Cervellin G. Influence of hemolysis on troponin testing: studies on Beckman Coulter UniCel Dxl 800 Accu-TnI and overview of the literature. Clin Chem Lab Med 2011;49(12):2097–2100
18 Lippi G, Musa R, Avanzini P, Aloe R, Pipitone S, Sandei F. Influence of in vitro hemolysis on hematological testing on Advia 2120. Int J Lab Hematol 2012;34(2):179–184
19 Lippi G, Ippolito L, Fontana R. Prevalence of hemolytic specimens referred for arterial blood gas analysis. Clin Chem Lab Med 2011;49(5):931–932
20 Favaloro EJ. More on preanalytical variables affecting platelet function testing using light transmittance aggregometry. Clin Chem Lab Med 2011;49(4):737–739
21 Lippi G, Fontana R, Avanzini P, et al. Influence of mechanical trauma of blood and hemolysis on PFA-100 testing. Blood Coagul Fibrinolysis 2012;23(1):82–86
22 Bonar R, Favaloro EJ, Adcock DM. Quality in coagulation and haemostasis testing. Biochem Med (Zagreb) 2010;20(2): 184–199
23 Lippi G, Salvagno GL, Montagnana M, Lima-Oliveira G, Guidi GC, Favaloro EJ. Quality standards for sample collection in coagulation testing. Semin Thromb Hemost 2012;38(6):565–575
24 Lippi G, Plebani M. Primary blood tubes mixing: time for updated recommendations. Clin Chem Lab Med 2012;50(4):599–600
25 Adcock Funk DM, Lippi G, Favaloro EJ. Quality standards for sample processing, transportation, and storage in hemostasis testing. Semin Thromb Hemost 2012;38(6):576–585
26 Lippi G, Plebani M. Continuous-flow automation and hemolysis index: a crucial combination. J Lab Autom 2012 (e-pub ahead of print). 10.1177/2211068212450014
27 Lippi G, Plebani M, Di Somma S, Cervellin G. Hemolyzed specimens: a major challenge for emergency departments and clinical laboratories. Crit Rev Clin Lab Sci 2011;48(3):143–153
28 Carraro P, Servidio G, Plebani M. Hemolyzed specimens: a reason for rejection or a clinical challenge? Clin Chem 2000;46(2): 306–307
29 Lippi G, Salvagno GL, Favaloro EJ, Guidi GC. Survey on the prevalence of hemolytic specimens in an academic hospital according to collection facility: opportunities for quality improvement. Clin Chem Lab Med 2009;47(5):616–618
30 Plebani M, Lippi G. Hemolysis index: quality indicator or criterion for sample rejection? Clin Chem Lab Med 2009;47(8):899–902
31 Martin-Toutain I, Jobic L, Mancic T, Ankri A. [Evaluation of the automated coagulation analyzer Sysmex® CS-2100i (Siemens)]. Ann Biol Clin (Paris) 2011;69(6):699–704
32 Molenaar PJ, Leyte A. Pre-acquisition system assessment of the Sysmex® Coagulation System CS-2100i and comparison with end-user verification; a model for the regional introduction of new analysers and methods. Clin Chem Lab Med 2011;49(9):1479–1489
33 Lippi G. Interference studies: focus on blood cell lysates preparation and testing. Clin Lab 2012;58(3-4):351–355
34 Lippi G, Musa R, Aloe R, Mercadanti M, Pipitone S. Influence of temperature and period of freezing on the generation of hemolysate and blood cell lysate. Clin Biochem 2011;44(14–15):1267–1269
35 Dimeski G. Effects of hemolysis on the Roche ammonia method for Hitachi analyzers. Clin Chem 2004;50(5):976–977
36 Lippi G, Mercadanti M, Aloe R, Targher G. Erythrocyte mechanical fragility is increased in patients with type 2 diabetes. Eur J Intern Med 2012;23(2):150–153
37 Lippi G, Banfi G, Buttarello M, et al. Recommendations for detection and management of unsuitable samples in clinical laboratories. Clin Chem Lab Med 2007;45(6):728–736
38 Fraser CG, Lippi G, Plebani M. Reference change values may need some improvement but are invaluable tools in laboratory medicine. Clin Chem Lab Med 2012;50(5):963–964
39 Favaloro EJ, Adcock DM. Standardization of the INR: how good is your laboratory's INR and can it be improved? Semin Thromb Hemost 2008;34(7):593–603
40 Lippi G, Favaloro EJ. Activated partial thromboplastin time: new tricks for an old dogma. Semin Thromb Hemost 2008;34(7): 604–611
41 Lippi G, Cervellin G, Franchini M, Favaloro EJ. Biochemical markers for the diagnosis of venous thromboembolism: the past, present and future. J Thromb Thrombolysis 2010;30(4):459–471
42 Favaloro EJ, Lippi G, Franchini M. Contemporary platelet function testing. Clin Chem Lab Med 2010;48(5):579–598
43 Linden MD, Tran H, Woods R, Tonkin A. High platelet reactivity and antiplatelet therapy resistance. Semin Thromb Hemost 2012;38 (2):200–212
44 Tripodi A. Problems and solutions for testing hemostasis assays while patients are on anticoagulants. Semin Thromb Hemost 2012;38(6):586–592
45 Walenga JM, Jackson CM, Kessler CM. Low molecular weight heparins differ substantially: impact on developing biosimilar drugs. Semin Thromb Hemost 2011;37(3):322–327
46 Lippi G, Franchini M, Favaloro EJ. Pharmacogenetics of vitamin K antagonists: useful or hype? Clin Chem Lab Med 2009;47(5): 503–515
47 Favaloro EJ, Lippi G. Laboratory testing and/or monitoring of the new oral anticoagulants/antithrombotics: for and against? Clin Chem Lab Med 2011;49(5):755–757
48 Favaloro EJ, Lippi G, Koutts J. Laboratory testing of anticoagulants: the present and the future. Pathology 2011;43(7):682–692
49 Favaloro EJ, Lippi G. The new oral anticoagulants and the future of haemostasis laboratory testing. Biochem Med (Zagreb) 2012;22 (3):329–341
50 Roß RS, Paar D. Analytisch und klinisch relevante Interferenzen in der Gerinnungsanalytik am Beispiel des MDA 180. Laboratoriumsmedizin 1998;22:90–94
51 Laga AC, Cheves TA, Sweeney JD. The effect of specimen hemolysis on coagulation test results. Am J Clin Pathol 2006;126(5):748–755
52 Lippi G, Montagnana M, Salvagno GL, Guidi GC. Interference of blood cell lysis on routine coagulation testing. Arch Pathol Lab Med 2006;130(2):181–184
53 Lippi G, Avanzini P, Pavesi F, et al. Studies on in vitro hemolysis and utility of corrective formulas for reporting results on hemolyzed specimens. Biochem Med (Zagreb) 2011;21(3):297–305
54 Tantanate C, Teyateeti M, Tientadakul P. Influence of plasma interferences on screening coagulogram and performance evaluation of the automated coagulation analyzer Sysmex® CS-2100i. Siriraj Medical Journal 2011;63(5):151–156
55 Lippi G, Avanzini P, Zobbi V, Ippolito L. Influence of mechanical hemolysis of blood on two D-dimer immunoassays. Blood Coagul Fibrinolysis 2012;23(5):461–463
56 Bauer NB, Eralp O, Moritz A. Effect of hemolysis on canine kaolin-activated thromboelastography values and ADVIA 2120 platelet activation indices. Vet Clin Pathol 2010;39(2):180–189

Downloaded by: Universita di Parma. Copyrighted material.

57 Franchini M, Targher G, Lippi G. Serum bilirubin levels and cardiovascular disease risk: a Janus Bifrons? Adv Clin Chem 2010;50:47–63
58 Grafmeyer D, Bondon M, Manchon M, Levillain P. The influence of bilirubin, haemolysis and turbidity on 20 analytical tests performed on automatic analysers. Results of an interlaboratory study. Eur J Clin Chem Clin Biochem 1995;33(1):31–52
59 Dimeski G. Interference testing. Clin Biochem Rev 2008; 29(Suppl 1):S43–S48
60 Quehenberger P, Kapiotis S, Handler S, Ruzicka K, Speiser W. Evaluation of the automated coagulation analyzer SYSMEX CA 6000. Thromb Res 1999;96(1):65–71
61 Appert-Flory A, Fischer F, Jambou D, Toulon P. Evaluation and performance characteristics of the automated coagulation analyzer ACL TOP. Thromb Res 2007;120(5):733–743
62 Fischer F, Appert-Flory A, Jambou D, Toulon P. Evaluation of the automated coagulation analyzer Sysmex CA-7000. Thromb Res 2006;117(6):721–729
63 Dorn-Beineke A, Dempfle CE, Bertsch T, Wisser H. Evaluation of the automated coagulation analyzer Sysmex CA-7000. Thromb Res 2005;116(2):171–179
64 Kroll MH. Evaluating interference caused by lipemia. Clin Chem 2004;50(11):1968–1969
65 Dimeski G, Jones BW. Lipaemic samples: effective process for lipid reduction using high speed centrifugation compared with ultracentrifugation. Biochem Med (Zagreb) 2011;21(1): 86–92
66 Lippi G, Aloe R. Hyponatremia and pseudohyponatremia: first, do no harm. Am J Med 2010;123(9):e17
67 Arambarri M, Oriol A, Sancho JM, Roncalés FJ, Galán A, Galimany R. [Interference in blood coagulation tests on lipemic plasma. Correction using n-hexane clearing]. Sangre (Barc) 1998;43(1): 13–19
68 Bai B, Christie DJ, Gorman RT, Wu JR. Comparison of optical and mechanical clot detection for routine coagulation testing in a large volume clinical laboratory. Blood Coagul Fibrinolysis 2008;19 (6):569–576
69 Lippi G, Salvagno GL, Lima-Oliveira G, et al. Interference of a regular standardized meal on routine coagulation testing. Poster presented at: Second Annual Joint Meeting SIBioC –SIMeL; October 27–30, 2009; Naples, Italy
70 McEnroe RJ, Burritt MF, Powers DM, Rheinheimer DW. Interference Testing in Clinical Chemistry; Approved Guideline—Second Edition. Wayne, PA: Clinical Laboratory Standards Institute; 2005. CLSI document EP7–A2
71 Vermeer HJ, Steen G, Naus AJ, Goevaerts B, Agricola PT, Schoenmakers CH. Correction of patient results for Beckman Coulter LX-20 assays affected by interference due to hemoglobin, bilirubin or lipids: a practical approach. Clin Chem Lab Med 2007;45(1): 114–119

Downloaded by: Universita di Parma. Copyrighted material.