Hemolysis interference in 10 coagulation assays on an instrument with viscosity-based, chromogenic, and turbidimetric clot detection

Ylva Hedeland1,2 | Christina M. Gustafsson2 | Zinah Touza1 | Peter Ridefelt1,2

Abstract

Introduction: Hemolysate in plasma samples from patients may cause misleading results in coagulation assays. Even though modern coagulation instruments often are equipped with modules that can detect hemolysis, icterus, and lipemia (HIL), studies that report the influence of these interferences are still limited. The present paper focuses on the influence of hemolysis on 10 coagulation assays.

Methods: Artificial hemolysis was created by freezing/thawing, and the hemolysates generated were added to pools of patient plasma. Pathological and normal levels were pooled separately. These spiked samples were analyzed on a STA R Max 2 instrument. The coagulation assays evaluated utilize clot, chromogenic, or immunoturbidimetric detection.

Results: Four of the evaluated assays were not influenced by hemolysis: fibrinogen, von Willebrand factor antigen, activated partial thromboplastin time, and factor VIII. Interestingly, normal and slightly elevated prothrombin time (INR < 2.0) was insensitive to hemolysis, whereas samples with a high INR (≥2.0) exhibited falsely high readings. The assays for antithrombin and fibrin D-dimer displayed an intermediate sensitivity to hemolysis. The most sensitive assay turned out to be anti-Xa, followed by protein C and protein S. For the anti-Xa assay, the results are decreased by 10% already at 0.5 g/L hemoglobin.

Conclusion: The present study shows that hemolysis affects several of commonly used coagulation assays. Since the sensitivity for hemolysis is dependent on the brand of the assay as well as the instrument and principle of measurement, it is necessary to evaluate the influence of each specific combination.

Keywords

coagulation, freezing, hemoglobin interference, hemolysis, pre-analytical error, serum indices
Sample handling in the pre-analytical and analytical phase is very important for the quality of analysis in laboratory diagnostics and eventually for the patient safety. The most common pre-analytical interference in the coagulation laboratory is spurious hemolysis caused by erroneous collection of the sample (40%), followed by pre-analytical clotting (29%) and inappropriate filling of blood tubes (28%). The interference of hemolyzed samples in coagulation assays is attributed to analytical as well as biological elements. The cell-free hemoglobin contributes to a higher absorbance at the detection wavelength(s), and the release of both intracellular components and thromboplastic membrane substances (e.g., tissue factor, proteases, phospholipids, and ADP) can activate platelets and blood coagulation.

Identification and reporting of hemolysis, as well as icterus, lipemia, and other potential interferences, are required by international standards like the ISO 15189. Traditionally, the identification and quantification of hemolysis have been performed by visual inspection of the color of serum or plasma. The automation of clinical laboratories with integration of pre-analytical and analytical workstations creates a risk that the identification of hemolyzed specimens is obstructed since automation solutions hide the tubes from visual examination. Even though many instruments used in general clinical chemistry nowadays are equipped with modules that detect and quantify hemolysis in the sample, such devices are rather novel on coagulation instruments. Additionally, the information about potential interferences supplied by the coagulation assay manufacturers is often relatively limited. Thus, the data from the manufacturers need to be supplemented by independent studies and in-house verifications. Furthermore, the setting of acceptability limits for hemolysis is of great importance for the interpretation of the results. Manufacturers often use a traditional generic value, usually a ±10% change from a nonhemolyzed sample, as their criterion for acceptability of interferences in their assays. However, it may be more suitable to set acceptability limits for interference that are based on biological and/or analytical variation data. Recently, the European Federation of Clinical Chemistry and Laboratory Medicine Working Group for Preanalytical Phase released recommendations for reporting of hemolyzed samples. These stated that the cutoff levels used should be predicted from the analytical variation and the within-subject biological variation. They also included a recommendation that analytical results with moderate interference from hemolysis should be released with an accompanying warning and comment, and that all results from samples with a clinically relevant hemolysis interference should be suppressed. The calculation of acceptability limits for clinical assays has been used based on different equations in the literature. The present study utilizes the formula from Fraser that has been suggested for determination of hemolysis interference.

The most commonly used methods for preparation of hemolyzed blood samples for interference studies is osmotic shock, freezing/thawing, and shearing with multiple needle aspirations. Recently, a comparative study of these three approaches was performed by Gidske et al. They concluded that the freeze/thaw method is to prefer since it includes lysis of leukocytes, platelets, and erythrocytes and is less laborious than the needle aspiration technique. Another approach is to ask for a second replacement sample for a hemolyzed patient sample. That design study will probably give the most correct answer, but it is laborious and the hemolysis level is hard to control for the laboratory.

Previous studies on hemolysis interference in coagulation assays have primarily dealt with the assays for prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and fibrin D-dimer while there is only a limited amount of studies performed on the assays for antithrombin, low-molecular-weight heparin, protein C (PC), protein S (PS), and factor VIII (FVIII). However, to the best of the authors’ knowledge, no previous hemolysis studies have been carried out on the assay for von Willebrand factor antigen (vWF). Furthermore, for each coagulation assay there are several different commercial kits as well as instrument from various vendors with different detection techniques (e.g., photo-optical and mechanical clot detection) on the market. Thus, in order to implement a cutoff value for an assay, the influence of hemolysis on the actual combination of kit and instrument needs to be evaluated.

The aim of the present study was to evaluate the interference of hemolysis on 10 coagulation assays on a Stago STA R Max 2 instrument, to facilitate the configuration of result reports, that is, to determine which hemolysis levels of the samples that need to be suppressed or released with a supplementary comment. All the studied assays are currently used in the routine analysis at Uppsala University Hospital, Sweden.

2 | MATERIALS AND METHODS

2.1 | Hemolysis interference

The hemolyzed blood samples were prepared based on the procedures described by Nagant et al. Briefly, nonhemolytic sodium citrate plasma (Greiner Vacuette, 3.2% sodium citrate) was anonymized, pooled, and frozen. The pooled plasma samples used were material left-over after the completion of diagnostic coagulation tests and were used in accordance with the approval by the Regional Ethical Review Board of Uppsala (no. 2001/367). The samples were collected based on previous laboratory results and pooled such that both pathological and normal levels were obtained for all assays studied. In total, 53 pools of plasma were created. Many of these pools were analyzed for more than one of the assays. The exception was the pooled samples containing LMWH that only was used for the anti-Xa assay.

Artificial hemolysis was created by freezing (−70°C, 24 hours) whole blood sampled in citrate tubes. Nonhemolyzed normal plasma was prepared from centrifuged (2400 g for 7 minutes) citrate blood. Both artificial hemolyzed blood and normal plasma samples were
obtained from healthy volunteers (ie, laboratory staff) that gave an informed consent for the investigation. Normal plasma and hemolyzed blood as well as pooled samples were stored at −70°C prior to analysis. The origin of the hemolysate and normal plasma within each pool was from the same volunteer. A range of hemolyzed samples with free hemoglobin from 0 to 15 g/L was prepared within each pool of plasma by addition of an increased amount of hemolyzed blood to a plasma pool aliquot, see Figure S1. To every aliquot, the same total amount of hemolysate and normal plasma (in different proportions) was added. The addition of hemolysate and nonhemolyzed plasma was 9% of the volume of the pool (ie, 160 µL to a 1600 µL pooled sample). The samples were immediately frozen at −70°C. Prior analysis, the samples were thawed at 37°C, vortexed, and centrifuged at 2400 g for 7 minutes.

### 2.2 | Assays

Three different types of coagulation methods were evaluated on the STA R Max 2 instrument (Stago): assays with viscoelastic detection of clotting time, chromogenic assays with photometric detection at 405 nm, and immunoassays with turbidimetric detection at 540 nm. The viscosity-based detection system was used for measurements of PT, APTT, and fibrinogen. The chromogenic assays were antithrombin, LMWH (anti-Xa activity), PC, and FVIII. The turbidimetric immunoassays were fibrin D-dimer, free PS, and vWF antigen. The FVIII reagent BioPhen FVIII:C was from Hyphen Biomed. All the other nine reagents were from Stago. For method details, see Table S1.

### 2.3 | Statistics

The results obtained for each analysis were tabulated in Excel (Microsoft). The sample without any addition of hemolyzed blood was set to 100%, and the bias was plotted as a function of hemolysis readings obtained from the Architect instrument (free hemoglobin in g/L). Interference by hemolysis was evaluated by comparison with the criterion ±10% change from the value in nonhemolyzed samples, often used by manufacturers, as well as the criterion suggested by Fraser. In the latter criterion, the interference should be less than \( \pm 1.96 \times (CV_a^2 + CV_w^2)^{1/2} \), where \( CV_a \) is the imprecision of the analytical method, and \( CV_w \) is the within-subject biological variation. Imprecision and biological variation were adopted from the Westgard database for all analytes except the drug LMWH.
where only the ±10% criterion was applied. For comparison, the analytical imprecision for all assays on the presently used instrument is also shown in Table 1. Maximum acceptable hemolysis was set to the lowest point where the regression line for hemolysis readings either exceeded the Fraser criterion or the 10% criterion. Both criteria were considered, since some methods receive unreasonably high limits using the Fraser approach, for example, D-dimer with ±51%, due to a very high within-subject biological variation, but also a relatively high analytical imprecision. Legends to figures include equations for regression lines based on all measurements, that is, both normal and pathological levels of the analyte.

A modified Student \( t \) test\(^2\) was used to evaluate whether the slopes of regression lines for pathological samples and samples within the reference range differed significantly.

3 | RESULTS

3.1 | Hemolysis readings on Architect vs STA R Max 2

The hemolysis readings from the Abbott Architect instrument were compared to the semi-quantitative index readings from the STA R Max 2, Figure 1. Most of the readings, 83%, gave a concordant result, but some discrepancies were noted. In general, the discrepant results were higher on the STA R Max 2 compared to the Architect readings.

3.2 | Clot assays

For fibrinogen, APTT, and PT (at normal or slightly elevated levels, ie, < 2.0 INR), no clinically relevant deviation caused by the hemolysis was observed in the range studied (≤15 g/L), Figure 2A-C. Interestingly, for samples with a PT(INR) ≥2.0 slightly increased readings were observed in PT assays, Figure 2D. The increase for PT(INR) ≥2.0 was statistically significant (modified student \( t \) test, \( P = .01 \)) compared to the normal and slightly elevated PT samples (<2.0). The readings for PT(INR) ≥2.0 exhibited a falsely high value (ie, exceeding the Fraser criterion) at 6 g/L hemoglobin, Table 1, which was in the H5 range, Figure 1. However, for all the other nine assays no significant difference was noted in the sensitivity for hemolysis when the regression lines for normal and pathological levels of the analyte were compared (data not shown).

3.3 | Chromogenic assays

Despite that fact that the detection wavelength of the chromogenic assays (405 nm) is around the peak maxima of hemoglobin,\(^1\) FVIII seems to be unaffected of hemolysis, Figure 3A. All the other three tested assays exceed the maximum allowed error in the studied range (≤15 g/L). The anti-Xa assay was most sensitive to hemolysis of all studied assays. The measured value of the LMWH pools that was analyzed with the anti-Xa assay was steeply decreased in hemolyzed samples and exceeded the manufacturer criterion already around 0.5 g/L, which is in the H2 range on the STA R Max 2 instrument, Figure 3B and Table 1. For PC, the readings were increased by hemolysis and exceeded the Fraser criterion at 3 g/L, that is, in the H4 range, Figure 3C. Antithrombin was less sensitive to hemolysis compared to PC, and too low readings were observed around 12 g/L in the H5 range, Figure 3D.

3.4 | Immunoturbidimetric assays

For the immunoturbidimetric assays, vWF was the only assay that seems to be unaffected by hemolysis, Figure 4A. Despite the higher detection wavelength (540 nm) compared to the chromogenic assays, the readings for PS and D-dimer were increased at higher hemolysis and the limit was exceeded around 8 and 6 g/L which is in the H5 and H4 range, respectively, Figure 4B-C and Table 1. For the D-dimer assay, the manufacturer criterion is applied, since the Fraser criterion gives too wide limits (±51%). It should also be noted that, due to the relatively high imprecision of the method, the coefficient of correlation for the D-dimer trendline is rather low (\( R^2 = .330 \)).

4 | DISCUSSION

The potential interference of hemolysis on PT and APTT assays has been the subject of several studies.\(^7\),\(^13\)-\(^17\),\(^27\),\(^28\) However, different combination of reagents and instrumentations makes the comparison between these studies cumbersome.\(^1\) One point to consider is the mode for clot detection. Several studies have used ACL TOP instruments from ILS\(^7\),\(^13\),\(^15\) or Sysmex or Dade Behring BCS instruments\(^7\),\(^16\),\(^27\),\(^28\) that utilize photo-optical detection, whereas the
Stago STA R instruments have a viscoelastic mechanical clot detection.\textsuperscript{7,14,17,18} Besides that, the content in the reagent kits varies among the manufacturers and many of the manufacturers offer more than one assay for the same analysis, for example, Stago offers four different assays for PT.

In some of the hemolysis studies in coagulation assays, a second sample has been requested when the initial sample from a patient was found to be hemolyzed.\textsuperscript{14,15} Even though this is a smart study design and give more representative samples, that approach is time-consuming and it is difficult to cover the whole concentration range of interest that are needed to determine a cutoff concentration where the samples should be suppressed. That is probably is the reason why in vitro induced hemolysis is the most commonly used method to study the influence of hemolysis. Also, the methods used to induce hemolysis in vitro vary. Thus, it is not surprising that the conclusions from different studies sometimes deviate.

The biological effects of hemolysis in the sample would affect the measurements on instruments with optical as well as mechanical clot detection, but the mechanical systems are less sensitive (or unsensitive) to optical interferences, that is, absorbance of cell-free hemoglobin.\textsuperscript{1,28} For APTT, several studies with photo-optical clot detection have concluded that hemolysis does affect the measurements.\textsuperscript{15,16,27} Among the studies of APTT with mechanical clot detection, a significant shortening,\textsuperscript{17} small insignificant shortening,\textsuperscript{14,18} and a prolongation of clotting times\textsuperscript{7} as well as no significant difference\textsuperscript{28} have been reported. In the present study with mechanical clot detection, a small but clinically irrelevant shortening of APTT was observed. This trend is in agreement with Florin et al,\textsuperscript{17} which is the only study mentioned above that use the same instrument and reagents as well as method to create artificial hemolysis. Thus, not only the instrument and reagents but also the mode for creating artificial hemolysis differed between the other studies mentioned above which might explain the dissimilarities in the conclusions.

In the present paper, fibrinogen was not affected by hemolysis, which is in accordance with the study from Woolley et al,\textsuperscript{14} that used the same reagents and instrument. Also, PT in the normal-low range (ie, INR < 2.0) was unaffected by hemolysis. Interestingly, the subgroup of PT(INR) ≥2.0 increased hemolysis was associated with a longer clot time. Even though the influence of the INR value was not discussed in the paper by Florin et al,\textsuperscript{17} their data hint that a higher clotting time might be more influenced by hemolysis. A STA R Max 2 instrument was used also in their study, but it should be stressed...
that the reagents differed. To the best of the author's knowledge, the influence on hemolysis on the SPA+ reagent is evaluated for the first time in the present study. Consequently, more studies are needed to confirm whether PT results are affected by the INR value in the individual patient, for example, for patients on warfarin treatment or with liver disease, and to elucidate which reagents that are sensitive to hemolysis.

Both the STA R Max 2 and the ACL TOP utilizes 405 nm for detection in their chromogenic methods. Jilma-Stohlawetz et al found that PC value was increased by increased hemolysis and suggested an acceptance cutoff at 0.6-2.0 g/L Hb. That observation is in accordance with the results in the present study, where an increase in PC value was observed, and the Fraser criterion was exceeded at 3 g/L Hb (Table 1). However, it should be emphasized that even though they used the same instrument as in the present study, the reagent kit differed. A falsely high PC value caused by hemolysis in the sample creates a risk of false classification of patients being investigated for PC deficiency after venous thrombosis.

The anti-Xa assay used for LMWH was even more susceptible to hemolysis and exceeds the acceptable limits (±10%) at 0.5 g/L hemoglobin. These results are in agreement with a previous study, that also observed a lowered anti-Xa activity at higher hemolysis levels. They used a Stago instrument and another reagent. The Stago anti-Xa activity reagent used in the current study could also be applied together with special calibrator for measurements of apixaban, rivaroxaban, edoxaban, and fondaparinux. Thus, it could be expected that also these assays for NOACs would be affected by hemolysis in a similar manner.

The chromogenic AT method showed a small decrease in values at higher degrees of hemolysis and exceeds the Fraser criterion at 12 g/L hemoglobin. This minor effect of hemolysis has also been observed on an ACL TOP instrument. The FVIII method, performed with a BioPhen reagent, did not show any sensitivity to hemolysis in the studied range (≤15 g/L). That is in agreement with previous findings by Laga et al.

Among the turbidimetric immunoassays, vWF seems to be unaffected by hemolysis. On the contrary, the value for D-dimer is...
increased by hemolysis and the acceptable limit (±10%) for the accuracy is exceeded above 6 g/L hemoglobin in that turbidimetric assay. This finding is in agreement with previous findings, for example, by Novelli et al.\textsuperscript{15} that requested a second replacement sample for the hemolyzed patient sample. However, the results from other studies on the influence of hemolysis in D-dimer assays diverge. Both positive bias\textsuperscript{15,16,28} and negative bias\textsuperscript{7,30} have been observed. Discrepant results have even been obtained in studies from the same group.\textsuperscript{16,31} That might be one explanation for the diverging results between the present study and Nagant et al.\textsuperscript{7} They used the same D-dimer reagents on a STAR Evolution instrument but the hemolysis in that study was created by addition of hemolyzed red cells (not hemolyzed whole blood) to the plasma sample.

A falsely high D-dimer value caused by hemolysis creates a major problem in clinical practice, since many requests for D-dimer come from emergency departments, and they are more prone to deliver samples with hemolysis.\textsuperscript{32,33}

The influence of hemolysis on PS was similar to that of the D-dimer, that is, the value was increased by increased concentration of hemoglobin. Interestingly, Jilma-Stohlawetz et al.\textsuperscript{19} observed decreased PS values in hemolyzed samples. However, that study used another reagent, and the hemolysis was induced through the shear principle.

In the present study, pooled patient plasma has been used to study the influence of hemolysis on 10 coagulation assays at different concentrations of the analyte, that is, at normal and pathological and normal levels. One limitation of the present study is that only one method for artificially creating hemolysis has been evaluated, and, as can be concluded from the literature, other methods might modify the results. Freezing/thawing was chosen since the resulting hemolysate also contains white blood cells and platelets from the disruption of cells. Even though mechanical disruption of blood cells by aspiration through fine needles might better mimic one of the major situations where hemolysis is created, it is complicated to obtain all hemolysis levels of interest by that method.

5 \hspace{1cm} \textbf{CONCLUSION}

The present study adds manufacturer-independent data on maximum acceptable hemolysis levels for 10 coagulation assays on a STA R Max 2 instrument. For six of the studied assays, a cutoff value for hemolysis has been suggested. However, it should be pointed out that the correlation coefficient $R^2$ for the linear regression is rather low for some assays (eg, D-dimer), thus making suggested cutoffs less reliable in some cases. That variability might be explained by a rather high imprecision of the assay as well as a high variability in the sample pools.

The methods based on viscosity measurement of clot formation, for example, fibrinogen and APTT, seem to be insensitive or less
influenced by hemolyzed samples than the assays with chromogenic and turbidimetric detection. The three assays that turned out to be the most sensitive to hemolysis were anti-Xa (LMWH), PS, and PC.

Since the sensitivity for hemolysis is dependent on the brand of the assay as well as the instrument and principle of measurement, it is necessary to evaluate the influence of each specific combination. The results for PT in the present study also reveal that the composition of the sample (i.e., the measured coagulation time or concentration of the measured analyte) might influence the hemolysis sensitivity for a certain assay.

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CONFLICT OF INTEREST

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ORCID

Ylva Hedeland https://orcid.org/0000-0002-0156-8242

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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