Methods for hemolysis interference study in laboratory medicine – a critical review

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ABSTRACT

Hemolysis represents an important source of error associated with the pre-analytical phase. Improving the protocols for detection, measurement, management of the parameters affected by the interference, and differentiation between hemolysis *in vivo* and *in vitro*, would favor a personalized management of hemolysis by increasing patient safety.

For this, it is essential to agree on the definition of "hemolysis". From this definition, a critical point is to establish cut-offs of hemolysis management for each analyte studied in the clinical laboratory.

Thus, in this review, the main methods described in the literature developed for obtaining a hemolysate are grouped, that simulate in controlled laboratory protocols what happens with a hemolyzed sample of a patient.

These methods are grouped into 3 categories according to their basis of lysing cells: freezing-thawing, osmotic shock and shear stress. In addition to development and improvement of methods for the study of

hemolysis, it is necessary to carry out comparative studies to determine which one offers the best capabilities. Harmonization of the methods will allow to include them in working guidelines. All these strategies will allow to move from managing hemolysis on whole-sample basis to customize it analyte by analyte.

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INTRODUCTION

Most of the errors associated with the total testing process occur in the pre-analytical phase (70-80%) (1,2). This phase includes sample collection, handling and transport, whose inadequate realization entails, inter alia, the possible appearance of analytical interferences, the main one being hemolysis (3). The presence of this interference in the samples affects the analytical determination of the biochemical tests, having as a consequence the need to recollect samples, delays in patient diagnosis and follow-up (crucial in the emergency laboratories), decreased patient safety, increased costs, …, etc. (4-6).

The hemolysis process is characterized by accelerated breakdown of the erythrocyte (RBC) membranes, releasing intra-erythrocyte content to the extracellular compartment (7). In this process not only hemoglobin is released, other components of the erythrocyte cytoplasm such as potassium, lactate dehydrogenase (LDH), or neuro-specific enolase (NSE) among other components.

All these elements can interfere with the measurements of the biochemical tests, and this interference will be more or less important depending on the magnitude assayed and the degree of hemolysis (8).

Due to the importance of knowing the degree of interference of the different levels of hemolysis over biochemical determinations, it is crucial

to develop and harmonize appropriate methods to establish as closely as possible cut-off points for the proper hemolysis management (9).

This step is very important for the subsequent development of rules that allow harmonized handling of hemolyzed samples. Thus, this review aims to provide insight into the methods available for studying hemolysis interference in clinical laboratories.

CAUSES OF HEMOLYSIS

The knowledge of the hemolysis degree of a sample is very important. This enables a different handling of the sample, appropriate to the type of hemolysis, and to perform corrective actions of the interferences.

Hemolysis can be due either to pathophysiological reasons or to causes exogenous to the patient. Thus, hemolysis can be classified into two large blocks:

- *Hemolysis* **in vivo**

   It is characterized by the breakdown of RBCs due to endogenous causes. Different degrees of anemia can be observed in the affected patient, able to increase the activity of the bone marrow as a compensatory mechanism (6). This premature rupture of RBCs may be due to antigen-antibody reactions, chemical reactions, hemolytic anemias, toxins or physical agents such as artificial heart valves, particularly mechanical ones (5). Less than 2% of the hemolysed samples detected in the clinical laboratory are due to *in vivo* processes (10). The handling of this type of samples will be aimed at knowing the patient's clinical situation and communicating the interferences to the clinician, in order to consider any possible treatments of the patient relevant to his/her pathophysiological situation.

- ### *Hemolysis* in vitro*:*

  In this case, the rupture of the RBCs is due to improper blood collection or sample handling (11). This kind of hemolysis may be due to phlebotomst using too thin needle size, inappropriate tubes mixing, or a too prolonged tourniquet time (12), as well as due to causes related to the transport or storage of the samples (13,14). In addition, this type of hemolysis is the one on which the clinical laboratory plays a more important role trying to reduce its incidence. To

do this, it must first be estimated and managed properly by including in the report the degree of interference, e.g. through comments, as well as by managing indicators to know their impact on the blood collection centers, strengthening the training where sample hemolysis rates are higher than the quality objectives set.

The knowledge of the type of hemolysis is important in order to perform the more appropriate management of the interfered parameters (Table 1).

Table 1	List of different causes of hemolysis occurring *in vivo* and *in vitro**
Causes of hemolysis *in vivo*	
Inherited	**Acquired**
Defects in hemoglobin	Immune-mediated causes
Defects of RBC membrane	Hypersplenism
Defective red cell metabolism	Burns
Glucose-6-phosphate dehydrogenase deficiency	Infections
Pyruvate kinase deficiency	Mechanical damage in circulation
Other RBC enzyme deficiencies	Inmunophenotyping errors in blood transfusion
	Drugs and toxins
Causes of hemolysis *in vitro*	
Operator-dependent	Handling of the specimen
	Transport of the specimen
Device-dependent	Sample processing
	Storage of the specimen

Table adapted from Lippi et al (7).

HEMOLYSIS MECHANISMS

Biochemical tests are interfered by RBCs breakdown as a result of the release of their cellular content. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) defines interference as "the systematic error of measurement caused by a sample component, which does not, by itself, produces a signal in the measuring system" (15). Interference (bias) in hemolysis processes will occur through four types of mechanisms described below:

- *Increase in the concentration of the constituent by intracellular release*

  Inside the red blood cell there are several analytes with higher concentration than in the surrounding fluid. Thus, their release as a result of hemolysis generates a positive interference on the relevant assays, causing overestimation of the real values. Potassium, LDH, AST, magnesium or phosphorus are the parameters that have the greatest difference between the intracellular medium of the RBC and the extracellular medium showing significant interference already at low hemolysis levels (7).

- *Chemical interference*

  In this mechanism, the interfering substance acts on one of the phases of the chemical reaction of the assay. In general, the interaction or competition of some substances released from the RBC, such as proteins or lipids, with reagents or reaction products will occur, being altered the concentration of the analyzed parameter (16). As examples of this interaction, the effect that hemoglobin exerts on the measurement of bilirubin, or the overestimation of creatin kinase by interference with adenylate kinase released by RBC.

- *Spectrophotometric interference*

  The wavelength at which the parameters that are measured spectrophotometrically are determined can overlap with the hemoglobin absorption spectrum. Thus, the assay of the analyte will undergo interference depending on the hemolysis degree. Both oxyhemoglobin and deoxyhemoglobin have a maximum absorption at 415 nm with a detection range between 320 nm and 450 nm, and between 540 nm and 589 nm respectively. The assays whose detection wavelength is between the above intervals will be affected, e.g. lipase, albumin and g-glutamyl transferase (GGT) (17,18). These analytes will be overestimated, while others such as alkaline phosphatase (ALP) will be underestimated when hemoglobin is degraded in alkaline medium (17).

- *Interference by dilutional effect*

  The analytes whose concentration is lower inside the RBC with respect to the extracellular fluids, will undergo further diminution when hemolysis occurs, due to dilutional effect. Thus, sodium, chloride and glucose are classic examples of this type of interference (7). Such an interference on sodium, which has a very narrow homeostatic regulation, can have important clinical relevance.

It is possible that, when hemolysis levels are very high, several described mechanisms happen simultaneously (17). The interference degree caused by hemolysis is not only important in spectrophotometric biochemical tests, but also in gasometric, hematocytometric, coagulometric and immunoassay tests.

MEASUREMENT OF HEMOLYSIS IN CLINICAL LABORATORY

Both types of hemolysis, *in vitro* and *in vivo*, are classically assessed by visual inspection comparing

the serum or plasma with a color scale after centrifuging the sample (19,20). This way of measuring hemolysis has important disadvantages (21), since it is a time-consuming method, therefore for laboratories with a high workload it is not very viable (5,22). Moreover, it is dependent on the observer thus on the degree of the observer training (23,19), and hardly standardizable to avoid intralaboratory biases (24). On the other hand, the assessment of hemolysis by visual inspection can be hindered by the presence of other interferers such as bilirubin, that adds color to the sample preventing a correct reading (25). Finally, this kind of hemolysis management could have consequences on patient safety (26).

To overcome the above difficulties in the assessment of hemolysis, the analyzers have progressively incorporated automated methods for the determination not only of hemolysis but also of icterus and lipemia (27). All these methods are based on performing a sample dilution and making multiple readings at different wavelengths using spectrophotometry (17). Farrell and Carter describe the characteristics of the automated HIL (hemolysis, icterus and lipemia) measurement methods of different analytical platforms currently available (17). With these methods, a qualitative or semi-quantitative determination is transformed to a quantitative determination allowing the development of control materials to implement both internal quality control programs (IQC) (28), as well as external quality control programs (EQA) for comparison among laboratories (29). But there are still challenges, making it difficult to implement this type of method in laboratories. Among the unresolved problems, Lippi lists several such as poor harmonization of detection techniques, non-standardization of the measurement units, improvement of the quality control systems available, and heterogeneity of the cut-offs between the different analytical platforms (30). Efforts are currently being made to try to solve these problems (28,

31), as well as demonstrating the low impact of any of them (32).

STUDIES FOR DETERMINATE HEMOLYSIS *IN VIVO*

Being able to differentiate hemolysis *in vivo* from hemolysis *in vitro* is presently a current challenge in the clinical laboratory, because of the impact more at the clinical level than at the analytical level. Currently there is no established consensus for the differentiation of hemolysis *in vivo* from hemolysis *in vitro*, but there are examples in the literature of strategies that try to improve differentiation (33). A described strategy is to compare different samples received in the laboratory of the same patient (34). When receiving multiple samples from a patient, the hemolysed sample is compared with the rest of the patient's samples; if all the samples show hemolysis levels above the set cut-off for hemolysis, a high probability exists of *in vivo* hemolysis. That should be confirmed with the medical history. On the other hand, if only one sample is hemolysed, an *in vitro* hemolysis can be suspected.

Lippi et al propose an algorithm for screening samples in which hemolysis is suspected *in vivo* (35). The hemolysis index value is transformed to g/L of hemoglobin. The samples with a free hemoglobin value greater than the upper reference limit (URL) and without suspected *in vitro* hemolysis will be tested further to confirm hemolysis *in vivo*. The development of mathematical models to allow screening of whole blood samples with hemolyzed *in vitro* has been proposed (36).

In addition to the algorithms, there are biomarkers that can help to determine hemolysis *in vivo.* These biomarkers have variable sensitivities and specificities for the study of hemolysis. Analytes such as potassium, LDH and aspartate aminotransferase (AST) increase markedly in hemolysis *in vitro* (34,37), however the concentration of potassium is not interfered always by hemolysis

in vivo (38). These samples can show increased potassium value for other physiopathological reasons, making screening difficult.

The decrease in haptoglobin concentration is considered the best marker to evaluate accelerated destruction of intravascular erythrocytes (39). In *in vitro* hemolysis this parameter is not affected since the resulting haptoglobin-hemoglobin complexes are rapidly eliminated from the circulation by monocytes and tissue macrophages CD163 receptor pathway (40). However, haptoglobin is decreased by hemolysis *in vivo*. But haptoglobin expression also decreases in liver function diseases which makes it difficult to use in the hemolysis assessment (41). Thus it should not be viewed as a gold standard (39).

Other typical *in vivo* hemolysis markers are indirect bilirubin and reticulocyte count increase, as well as decreased erythrocyte indices such as Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH). But none of the biomarkers described are going to be affected only by the presence of hemolysis, therefore it is necessary to develop specific biomarkers to help us differentiate *in vivo* hemolysis from *in vitro* hemolysis.

Currently there is no established consensus to distinguish hemolysis *in vivo* and *in vitro* (7). Thus it is necessary to continue working on the development of algorithms, that allow us to differentiate the origin of the hemolysis, due to its clinical significance.

ASSESSMENT OF *IN VITRO* HEMOLYSIS

Due to the high impact that *in vitro* hemolysis has on the assays in the clinical laboratory, homogeneous procedures are needed that allow us to determine cut-off points for the proper handling of hemolyzed samples. There is currently a high degree of heterogeneity in these studies (42,18). Thus we can decide whether or not to report the result obtained (43,44), or which is the most

appropriate handling procedure depending on the parameter. In 2018, the Working Group for Preanalytical Phase (WG-PRE) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) (45), published a document of recommendations for the harmonized management of the results of hemolyzed samples, in order to know the cut-offs for each analyte in each analytical platform, since the values are dependent on method and instrument (46). Thus, an individualized management of each parameter can be achieved, all not being influenced in the same way by the same degree of hemolysis (46).

All the methods described in the literature and used for *in vitro* hemolysis studies are based on three fundamental strategies:

- **Sample Freezing:** i.e. Freezing of the sample or freeze-thaw cycles to breakdown the cells.

- **Osmotic shock:** i.e. Rupture of the cells when resuspended in hypotonic medium, usually distilled water.

- **Shear stress:** i.e. Mechanical rupture of the cells by passing them through a needle.

In this section, different strategies for estimating the degree of interference by hemolysis, of the analytes to be measured in the clinical laboratory will be described.

CLASSICAL METHODS FOR HEMOLYSIS INTERFERENCE STUDY

The key point is to obtain a hemolysate that adequately represents the working conditions. Different strategies for obtaining hemolysate have been described. Having a hemolysate that mimics most closely hemolyzed patient samples will allow the most appropriate handling of the samples. Lovelock describes a method for obtaining hemolyses based on the freezing of the sample (47), e.g. starting from the whole blood sample and freezing it or freezing washed red

blood cells. Freezing is performed over a period of 30 min, which is insufficient to break the platelets and leukocytes if whole blood is used, requiring longer periods of freezing (48).

Twenty years later, Meites published a modified method based on the Lovelock method (49). The Meites method is the classic method used to obtain hemolysis recommended by different guides (50). In this method a hemolysate is generated which will be used to add it to serum or plasma samples of patients where interference is studied. Anticoagulated whole blood is centrifuged to obtain the sedimented RBCs and washed 3 times with isotonic saline. The cells are frozen overnight in water and thawed at room temperature next day. It is centrifuged to remove cell debris and the hemoglobin concentration in the hemolysate is determined. Finally, hemolyzate is added to serum samples or plasma at different concentrations. Freezing-thawing of these methods breaks the blood cells and centrifugation removes cell debris, similar to the handling of the samples after venipuncture. To try to improve this method, studies have been done modifying the conditions of freezing temperature and freezing time (51).

It is important to consider that in these methods, starting from centrifuged and washed RBCs, the contribution that the breakdown of leukocytes and platelets have on the interference caused by hemolysis is lost.

Another alternative is the method described by Glick et al (52). This method starts with washed erythrocytes that are only going to be lysed using distilled water (osmotic shock). The hemolysate is cleaned by filtering on the glass wool and subsequent centrifugation. Like the Meites method, it has the limitation that only the breakdown of RBCs contributes to hemolyzing. Other method, less used, is proposed by Frank et al (53). This method also starts with washed erythrocytes to which distilled water and detergent are added to favor its rupture.

NEW APPROACH FOR HEMOLYSIS INTERFERENCE STUDY

Based on the work of Meites and Glick, different strategies have been developed with the aim of achieving better hemolysates. Some of them are new strategies and others are modifications of those already available. The classical methods were based on freezing strategies (Lovelok and Meites) or osmotic shock (Glick) using distilled water. In 2004, Dimeski described a new method based on passing the anticoagulated whole blood sample through a needle several times to cause the breakdown of cellular components (54). The number of times the sample is passed through the needle determines the degree of hemolysis achieved.

Lippi et al (55) made a modification of Dimeski method, by standardizing the number of times the sample was passed through the needle (1 to 4 times), the type of syringe used (insulin type, 0.5 mL), as well as the thickness of the needle (30 gauge). The sample was centrifuged to remove the debris before determining the amount of free hemoglobin. As in the freeze-thaw models, with this method the erythrocytes were not selectively broken as in the osmotic shock models, since all blood cells in the sample (erythrocytes, leukocytes and platelets) are broken (9). In addition, an advantage of Dimeski's method is that by passing the sample through a needle simulates what is happening when collecting a patient's sample (56). The Lippi modified Dimeski method has been used for studies of hemolysis interference in parameters such as gasometry (56), coagulation (57), and hematimetry (58). Using this method, Lippi et al obtained the following conclusions (58):

- The hematology equipment used does not discriminate erythrocyte hemoglobin to free hemoglobin.

- A decrease in the RBC and hematocrit count due to mechanical rupture is

observed, confirmed by a parallel increase in LDH and hemolytic index.

- A significant decrease in the number of total leukocytes is not detected

- Problems were observed in leukocyte formula assessment due to interference with RBC and platelet clumps.

In this type of samples, the main interference is not associated with the presence of free hemoglobin, but with the rupture of the different blood cell lines, leading to significant errors in the cell count. In hematological samples it is not appropriate to apply the methods of osmotic shock and freezing because all the cells in the sample would be broken and could not be assessed in the hematological counter.

In methods employing whole blood (gasometry and hematocytometry), it has been proposed to perform a previous screening of the samples by collecting an aliquot, centrifuging and determining visually the degree of hemolysis (59). This approach, especially for a laboratory with high workloads, would increase response times and increase workloads on the whole. These disadvantages would be more critical in emergency laboratories where the number of samples of gasometry and hematocytometry is high.

Recently Delgado et al, based on freezing-thawing methods for hemolysis studies, proposed two different strategies (60):

- ### *Strategy 1*

   Collection of two anticoagulated whole blood tubes. One was centrifuged and the supernatant (plasma) was collected. The other tube was frozen-thawed 3 times, centrifuged and the supernatant was collected. The two supernatants (plasma) obtained in different proportions were mixed.

- ### *Strategy 2*

   Collection of two anticoagulated whole blood tubes. One of them was centrifuged, the supernatant (plasma) was removed and the pellet was frozen-thawed (without using distilled water). The other tube was centrifuged and the supernatant (plasma) was collected. Different volumes of hemolysate to the plasma of the second tube were added.

In both strategies a negative interference (decrease in concentration) was observed in the determination of Na$^+$, but strategy 1 presented better repeatability. The interference on Na$^+$ was due to dilution, therefore not including dilution with water in these strategies allowed a better assessment of Na$^+$ interference compared to previous studies that did include the dilution step (61).

In order to evaluate the suitability of the methods in the study of hemolysis interference, and to verify which one has better performance, it is necessary to carry out comparison studies between them. Studies have been carried out to compare the methods of obtaining hemolysate by osmotic shock and shear stress. In both cases they have been studied to determine the effect of hemolysis on the determination of ammonium (54,62), showing a better detection of interference using the osmotic shock method.

These studies did not include the freezing-thawing method, so Gidske et al (63) first compared the three main methods, namely: freezing, osmotic shock and shear stress. They studied 10 biochemical parameters on two different analytical platforms based on liquid chemistry or alternatively on dry chemistry assays. LDH was the parameter that provided more information, the interference observed was greater in the freezing-thawing and shear stress methods than the osmotic shock methods. The shear stress method produced the breakdown of erythrocytes,

platelets and leukocytes but it was a laborious method, with a high variability in the release of hemoglobin. On the other hand, the freezing method is simpler, with less variability and the three cell lines are also broken. In this study, freezing-thawing method was recommended as the most appropriate for obtaining hemolysates in hemolysis interference studies.

OTHER METHODS

In addition to the methods described above, other methods for the breakdown of blood cells based on different physical principles have been published. Yücel et al described the mechanical rupture of cells by stirring whole blood with a metallic bar (64). In another paper, Larga et al proposed hemolysis using tissue homogenization equipment (65). For the realization of these methods, equipment is needed that is not normally available in clinical laboratories, limiting its use in hemolysis interference studies.

Table 2 summarizes the characteristics of the methods for performing *in vitro* hemolysis studies in the clinical laboratory.

Table 2	Main methods reported for obtaining in *vitro* hemolysates and their characteristics			
Method	**Basis**	**Sample**	**Use water**	**Cell type break**
Lovelock (47)	Freezing-thawing	Whole blood/ Washed Erythrocytes	No	Erythrocytes-Leukocytes-Platelets/ Erythrocytes
Meites (49)	Osmotic shock/ Freezing-thawing	Washed Erythrocytes	Yes	Erythrocytes
Glick et al. (52)	Osmotic shock	Washed Erythrocytes	Yes	Erythrocytes
Frank et al. (53)	Osmotic shock	Washed Erythrocytes	Yes (+detergent)	Erythrocytes
Dimeski (54)	Shear stress	Whole blood	No	Erythrocytes-Leukocytes-Platelets
Dimeski adapted by Lippi et al. (58)	Shear stress	Whole blood	No	Erythrocytes-Leukocytes-Platelets
Delgado et al. (60)	Freezing-thawing	Whole blood	No	Erythrocytes-Leukocytes-Platelets/ Erythrocytes

CONCLUSIONS

The harmonization/standardization of the processes performed in the clinical laboratory is an important objective to reduce variability and generate high quality results. The proper handling of hemolysis interference, in addition to correct analytical measurements, is particularly influenced by the determination of precise cut-offs for each analyte studied.

At first, it should be defined "hemolysis", i.e. whether it is the rupture of only the erythrocytes or of all blood cells (erythrocytes, leukocytes and platelets). Following a classical approach, e.g. Meites et al and Glick et al, only washed erythrocytes are employed. On the other hand, more recent studies, such as Lippi et al, take into account the contribution of the three cell lines, and Delgado et al and Gidske et al test different strategies, concluding that methods based on whole blood samples are more suitable for interference studies by hemolysis. Once the term "hemolysis" is defined, it is necessary to develop consensus protocols for the study of interference by hemolysis, by exchanging results between laboratories. In this review we have described the three groups of procedures currently used: freezing-thawing, osmotic shock and shear stress. Among these, a lot of heterogeneity in the method used to obtain hemolysate is reported in literature. To solve the problem it is necessary to carry out comparative studies of the available methods, such as that carried out by Gidske et al, in order to select the most appropriate protocol.

Another possible approach, as done by Delgado et al., is based on a method already described that introduces modifications allowing a better assessment of the degree of interference in the samples. All these studies should be extended to all the currently available analytical methods as well as to all analytical platforms. The availability of consensual method(s), could allow their introduction into international guides, such as CLSI, harmonizing the protocols for carrying out hemolysis studies. The proper definition of the methods to be used in each situation would allow to obtain precise cut-offs independent of the method used to determine the degree of hemolysis interference. Thus, the management of hemolysis through interpretative comments proposed by the WG-PRE of the EFLM, would allow better assessment of each parameter that is being interfered and to obtain information useful for either the management of the clinical laboratory or the interpretation of the result at the clinical level.

Finally, a need is apparent to continuously progress in developing algorithms to differentiate hemolysis *in vivo* from *in vitro,* wieved as a challenge for the future in the clinical laboratory. In summary, with the harmonization/standardization of all these strategies, the integral management of hemolysis as a whole (clinical-analytical) will be favored with a notable increase in patient safety.

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