Hemolysis interference studies: the particular case of sodium ion

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Background
Despite many studies assessing hemolysis interference in almost every clinically relevant magnitude, sodium has poorly been assessed. Our aim was to evaluate hemolysis interference on plasma sodium, using different strategies of hemolysis preparation, at different baseline sodium ion concentrations and bias specifications.

Methods
Two different strategies were used for the preparation of hemolysis from lithium heparin blood samples. Repeatability was calculated at two levels for each strategy and interferograms were outlined for both approaches at sodium concentrations between 130-145 mmol/L. Results were interpreted according to different specifications: reference change value, RiLiBÄK, Westgard’s database, RCPA-QAP and CLIA.

Results
The coefficients of variation of the hemolyzed samples using the first strategy were lower than for the second strategy (0.23-0.78% vs 0.57-48.6%, for 0.2 g/dL...
free Hb and 0.28-0.44% vs 0.40-135.1%, for 0.9 g/dL free Hb). Statistically significant differences were seen when comparing the slopes of the pairs of interferograms at each sodium concentration obtained by both strategies (p<0.001 for 130 mmol/L; p=0.068 for 135 mmol/L; p=0.002 for 140 mmol/L and p=0.001 for 145 mmol/L). Hemolysis cut-off values were generally independent of the sodium concentration.

**Conclusions**

Reproducibility of hemolysate preparation is procedure-dependent. A greater standardization is needed for the preparation of a true hemolysate to better quantify the degree of interference of clinically relevant analytes, especially those with higher complexity such as sodium. We found a concentration-independent cut-off value for the hemolysis index that allows the establishment of a single and robust value in every laboratory, according to their quality specifications.

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**INTRODUCTION**

In the preanalytical phase, the **in vitro** lysis of red blood cells (hemolysis), which implies a release of hemoglobin and other intraerythrocyte elements, is the main cause of interference and rejection in the biochemical analytical methods worldwide (1–3). **In vitro** hemolysis depends mainly on blood sample drawing techniques and subsequent treatment (agitation, transportation, storage), whereas **in vivo** hemolysis may have at least 50 causes (4,5), including Gram-positive bacteria, parasites, toxins or autoimmune disorders. Genetic disorders such as sickle-cell disease or glucose-6-phosphate dehydrogenase deficiency may also lead to hemolytic crises with high free hemoglobin levels in blood. There are two central mechanisms of interference by hemolysis in clinically relevant tests: spectral (especially in spectroscopic methods, due to an overlapping of absorption spectra) and chemical (due to a release of components from red blood cells which alter the **in vivo** concentration of the analyte) (6). Other hemolysis-derived interfering mechanisms may be due to other causes (e.g. magnesium in the measurement of total calcium concentration, or adenylate kinase in the measurement of creatine kinase activity).

A great number of studies have assessed the effect of hemolysis on almost every clinically relevant analyte. Towards a minimization of variability and a higher reproducibility, guidelines have been published both for the performance of such studies and for the **in vitro** simulation of hemolysis and the handling and processing of such blood specimens (7–10). The quantification of the degree of hemolysis is also fundamental for the proper management of samples and test results (11,12).

In spite of their almost universal applicability for biomarkers in laboratory medicine, there are a few exceptions still needing a thorough examination, and the paradigm of such exceptions is sodium ion. As previously reported, in vitro hemolysis is known to negatively interfere with sodium due to a diluting effect (13,14), as the intracellular concentration of sodium is significantly lower than the concentration in serum or plasma. The degree of hemolysis in a sample is frequently assessed by measuring the free hemoglobin in serum or plasma.

The preparation of a true hemolysate is crucial for the performance of studies assessing hemolysis interference. This term refers to the absence of unhemolyzed red blood cells or other intact cells after the preparation of hemolysate. One of the procedures most commonly was first described by Meites (15), and includes a water-dilution step before freezing and thawing an anticoagulated blood sample. Other useful
strategies in literature include microwave radiation, ultrasounds or mechanic lysis (16).

Nevertheless, the preparation of valid hemolysate for the study of sodium in serum or plasma should not have a water-addition step, as it would decrease the concentration of the ion in the solution, hence altering the (direct) relationship with the hemolysis index. According to literature, intraerythrocyte sodium concentration is 10-15 mmol/L (17,18), whereas sodium concentration in distilled water is negligible. As a result, the water-dilution step in the preparation of a hemolysate would alter the relationship between hemoglobin (hemolysis index) and sodium, as well as with any other intraerythrocyte biomolecule. If including this step, the greater water volume added for the preparation of the hemolysate, the greater interference observed (negatively). The equilibration with distilled water would impede the detection of the strictly negative effect of hemolysis.

Our aim was to comprehensively assess the magnitude of hemolysis interference of plasma sodium, using different strategies of hemolysate preparation, different baseline sodium ion concentrations and different bias specifications in their interpretation.

**METHODS**

Two different study procedures were suggested in this study to parallelly assess and quantify the magnitude of interference of hemolysis on plasma sodium (Figure 1).

A total of 40 volunteers were recruited for the performance of this study: 20 for the first approach, and 20 for the second.

The first approach consisted in blood extraction into two simultaneous 3.5-mL lithium heparin tubes without gel from each volunteer (ref. 368884, BD Vacutainer). One of them was directly centrifuged (10min 1500g; Sample A) while the other was previously frozen-thawed 3 times to induce hemolysis and subsequently centrifuged (Sample B). Both plasma samples A+B were mixed in different proportions, starting from [1000 µL A + 0 µL B] to [1000 µL A + 200 µL B] (greater proportions of B yielded excessive hemolysis, not quantifiable by the analyzer). Sodium ion was measured by indirect potentiometry, whereas hemolysis index was analyzed by dichromatic spectrophotometry at different wavelengths and calculated using an algorithm (Architect c16000 platform, Abbott Diagnostics, USA). Sodium in sample A (pure) was taken as reference. The experiment was performed at four concentrations of sodium: approximately 130, 135, 140 and 145 mmol/L. None of the samples was seen to by hyperlipidemic.

The second approach consisted in the simulation of hemolysis by removing the supernatant and the buffy coat of a plasma heparin tube, freezing-thawing it 3 times and further centrifuging it (10min 1500g). No addition of distilled water or washing step was carried out. Pools of lithium heparin plasma were prepared at different sodium concentrations (approximately 130, 135, 140 and 145 mmol/L) using plasma from 5 different participants for each, and aliquoted into 1-mL tubes. Increasing volumes of the hemolyzed supernatant (5, 10, 20, 25, 30, 35 and 40 µL) were added to each aliquot, which were further centrifuged to remove possible intact red blood cells.

Repeatability of both strategies was assessed by measuring 10 times the hemolysis index at 5-minute intervals in 10 samples, at two different hemolysis indices (approx. 0.2 and 0.9 g/dL) for each strategy in different days, and calculating the coefficients of variation.

Interferograms were outlined for both approaches and different initial sodium concentrations, according to guidelines (7).
The critical hemolysis index causing interference was evaluated in every interferogram as the difference from the baseline value, and established according to five different performance specifications:

- the reference change value in our laboratory (RCV), which integrates the within-subject biological variation (0.6%) and our analytical coefficient of variation (0.67%);
- the RiLiBÄK specification of 3%;
- the desirable quality specification of 0.73% for total error on the Westgard database (available at: https://www.westgard.com/biodatabase1.htm);
- the allowable limit of performance of 2% by the Royal College of Pathologists of Australasia (RCPA-QAP) (available at: https://www.westgard.com/rcpa-biochemistry.htm); and
- the CLIA specification of ±4 mmol/L (available at: https://www.westgard.com/clia.htm).

The Shapiro-Wilk’s test was used to assess normality, and outliers were removed using the Reed/Dixon’s test. The Fisher’s F-test was carried
out to compare variances and the Student’s t-test was used in order to compare the slopes and intercepts of both hemolysis-preparing strategies at each sodium concentration. Statistical significance was set at 5%. The software SPSS v.20 was used for all statistical analyses.

RESULTS

The coefficients of variation for the repeated measures were 0.26-0.78% and 0.28-0.44% for hemolysis levels obtained by the first strategy (parallel extraction and mixing in different proportions), and 0.57-48.6% and 0.40-135.1% for the samples obtained using the second strategy (hemolysate generation and subsequent addition to a normal plasma) (Table 1).

Sodium concentration was seen to decrease with hemolysis in every concentration assessed, independent of the hemolysis strategy (Figure 2). When comparing the slopes of the pairs of interferograms at each sodium concentration obtained by both strategies, statistically significant differences were detected (Table 2).

As outlined in Table 3, when the approach based on hemolysis generation in whole blood, centrifugation and mixing in different proportions with a paired normal plasma was used (strategy 1), the mean hemolysis index exceeding the RCV specification for a sodium concentration of 130 mmol/L was 0.95 g/dL hemoglobin (CI 95%: 0.88-1.02), while this critical value is reduced when a more strict limit is used, such as the one suggested on the Westgard database.

| Table 1 | Repeatability (coefficients of variation) of hemolysis index obtained by different strategies |
|---------|--------------------------------------------------------------------------------------------------|
|         | Plasma [Na⁺] (mmol/L) | Low Hemolysis Assay (0.2 g/dL Hb) | High Hemolysis Assay (0.9 g/dL Hb) |
| Strategy 1 | 144.1 | 0.26-0.78% | 0.28-0.44% |
| Strategy 2 | 144.3 | 0.57-48.6% | 0.40-135.1% |

| Table 2 | Comparison of slopes and intercepts of interferograms obtained at a specified sodium concentration (y: deviation from baseline as %; x: free hemoglobin in g/dL) |
|---------|--------------------------------------------------------------------------------------------------|
| Plasma [Na⁺] (mmol/L) | Regression equation strategy 1 | Regression equation strategy 2 | p-value (slope) |
| 130 | y = –0.003x+0.088 | y = –0.004x–0.136 | <0.001* |
| 135 | y = –0.002x–0.198 | y = –0.003x–0.076 | 0.068 |
| 140 | y = –0.002x–0.272 | y = –0.003x+0.013 | 0.002* |
| 145 | y = –0.002x–0.190 | y = –0.004x–0.046 | 0.001* |

* Significant at p<0.05
Figure 2  Interferograms for different sodium concentrations using both hemolysis preparing strategies

Percentage deviation from sodium baseline value according to increasing hemolysis in the samples.
Table 3  Hemolysis interference cut-off on plasma sodium for different bias specifications

|                      | Free hemoglobin (g/dL)                                                                 |
|----------------------|----------------------------------------------------------------------------------------|
|                      | [Na⁺] 130mmol/L | [Na⁺] 135mmol/L | [Na⁺] 140mmol/L | [Na⁺] 145mmol/L |
| CI for samples used  |               |               |               |               |
| in Strategy 1        | (130.7-133.0)  | (136.2-137.7)  | (140.3-142.7)  | (145.6-148.5)  |
| CI for samples used  |               |               |               |               |
| in Strategy 2        | (131.7-133.1)  | (135.7-137.7)  | (139.3-142.5)  | (145.8-147.0)  |
| RCV (2.5%)           | Strategy 1     | 0.95           | 0.96           | 0.99           | 1.04           |
|                      | (0.88-1.02)    | (0.88-1.04)    | (0.90-1.08)    | (0.95-1.13)    |
|                      | Strategy 2     | 0.61           | 0.86           | 0.77           | 0.70           |
|                      | (0.56-0.67)    | (0.80-0.92)    | (0.70-0.84)    | (0.62-0.78)    |
| RiLiBÄK (3%)         | Strategy 1     | 1.13           | 1.17           | 0.93           | 0.84           |
|                      | (1.05-1.22)    | (1.07-1.26)    | (0.84-1.01)    | (0.74-0.94)    |
|                      | Strategy 2     | 0.74           | 1.04           | 0.93           | 0.84           |
|                      | (0.68-0.80)    | (0.97-1.12)    | (0.84-1.01)    | (0.74-0.94)    |
| Westgard (0.73%)     | Strategy 1     | 0.30           | 0.22           | 0.20           | 0.24           |
|                      | (0.38-0.49)    | (0.30-0.45)    | (0.27-0.46)    | (0.33-0.48)    |
|                      | Strategy 2     | 0.15           | 0.23           | 0.23           | 0.21           |
|                      | (0.18-0.32)    | (0.31-0.42)    | (0.26-0.42)    | (0.23-0.40)    |
| RCPA-QAP (2%)        | Strategy 1     | 0.88           | 0.84           | 0.81           | 0.86           |
|                      | (0.81-0.94)    | (0.77-0.90)    | (0.74-0.88)    | (0.78-0.93)    |
|                      | Strategy 2     | 0.56           | 0.76           | 0.65           | 0.59           |
|                      | (0.51-0.61)    | (0.70-0.81)    | (0.59-0.71)    | (0.52-0.66)    |
| CLIA (±4mmol/L)      | Strategy 1     | 1.17           | 1.17           | 1.17           | 1.17           |
|                      | (1.08-1.26)    | (1.08-1.26)    | (1.06-1.27)    | (1.06-1.28)    |
|                      | Strategy 2     | 1.17           | 1.17           | 1.17           | 1.17           |
|                      | (1.08-1.26)    | (1.08-1.26)    | (1.06-1.27)    | (1.06-1.28)    |

Confidence intervals are shown in parentheses. Abbreviations: RCV: reference change values; RCPA-QAP: Royal College of Pathologists of Australasia Quality Assurance Program.
for desirable quality specifications for total error (0.30 mg/dL hemoglobin). When the approach based on the preparation of hemolysate and small-volume addition into normal samples was followed (strategy 2), lower hemolysis cut-off values were obtained at most concentrations. At a sodium concentration of 130 mmol/L, a hemolysis of 0.61 g/dL Hb was shown to interfere when the RCV specification was used, while small amounts as 0.15 g/dL Hb were seen to interfere when the Westgard’s specification was followed.

There is no fixed tendency in the hemolysis cut-off at different sodium concentrations. Hemolysis cut-off values are generally independent of the baseline sodium concentration.

DISCUSSION

There is currently an objective and evident improvement in patient safety thanks to the automatization in the measurement of hemolysis index in the clinical laboratories, which replaces behind the visual inspection for the decision upon their adequacy (19). The optimal hemolysis index cut-off value for each clinically relevant test is method- and instrument-dependent, and always subject to the previously defined quality specifications in every specific laboratory (5). In hemolysis interference studies, the procedure for the preparation of hemolysate is of utmost importance (20).

Hemolysis-preparing strategies

In our study, two different strategies were used to assess hemolysis interference at different plasma sodium concentrations. The first strategy, based on a parallel blood drawing, freeze-thawing one whole-blood sample, centrifuging and mixing in different proportions with non-hemolyzed plasma, showed a better repeatability than the second strategy, based on hemolysate generation and subsequent addition to a normal plasma. This better repeatability yields a greater reproducibility and robustness of the first strategy.

Hemolysis index cut-off establishment

Many studies may be found in literature assessing hemolysis interference for chemistry analytes, although sodium ion is only assessed in very few of them.

Steen and colleagues (21) assayed two different sodium concentrations (127 and 140 mmol/L), using an hemolysate prepared by osmotic disruption with distilled water, and could not detect any significant difference in the values, thus interpreting an absence of interference. As stated, the inclusion of a water-dilution step in the preparation of the hemolysate could most probably add a bias in the results.

Another approach by Lippi et al (22) followed a freeze-thaw procedure for hemolysis preparation and, with a critical difference set at ±0.3%, found that even small amounts of hemolysis (0.016 g/dL of hemoglobin) could interfere in the measurement of sodium (seen at [Na+] = 140.1±1.5 mmol/L).

Another study by Saldaña and collaborators (23) introduced a washing step with NaCl in the preparation of the hemolysate. After correcting for the dilution, an hemoglobin concentration of 0.21 g/dL was shown to induce interference.

A third study evaluated one single sodium concentration (146 mmol/L) and found a deviation of 2% from baseline when hemolysis was 0.66 g/dL. Greater concentrations of free hemoglobin were not assessed (24).

In our study, given the interval of sodium concentrations, we found a concentration-independent cut-off value for the hemolysis index, at different bias specifications. This allows the establishment of a single cut-off value for hemolysis in every laboratory, according to their quality specifications.
Apparent hyponatremia

There are varied and important clinical implications derived from a falsely reduced plasma sodium result due to hemolyzed samples, which is the main cause of preanalytical rejection of samples (14, 25). In addition, there is no consensus whether sample transportation to the laboratory may influence the degree of in vitro hemolysis, whether by pneumatic tube or not (26–28).

Hyponatremia, defined as a sodium concentration <135 mmol/L, is reported to have an incidence between 4-19% of outpatients, being also associated with a mortality increase (29). After the diagnosis of a in vivo hyponatremia, a comprehensive differential diagnostic study is essential to determine the exact etiology, including volemia, sodium clearance and serum osmolality, among other biochemical tests (30). The treatment of hyponatremia needs to be directed to correct the etiologic cause and restore blood sodium levels with a rate 6-12 mmol/L in the first 24 hours, as a greater rate would trigger an osmotic demyelination (31). Therefore, robust and reliable sodium results by the laboratory are crucial not only for the diagnosis of electrolyte imbalance disorders, but also for the decision of treatment strategies and follow-up.

The wide range seen in previous bibliography for the establishment of a hemolysis cut-off for sodium interference highlights the challenging aspect of this cation. Our study is the first to comprehensively assess the impact of hemolysis on plasma sodium, including two strategies for sample preparation, several sodium concentrations (130 to 145 mmol/L), and interpreting such results according to different deviation specifications. The inclusion of different specifications in the establishment of a cut-off value makes our approach of greater applicability for laboratories worldwide. The main limitations of our study relate to the use of a single analytical platform, given that hemolysis index is measured by different spectroscopic methods and mathematical algorithms among clinical laboratories, as well as the low number of samples included, although in line with previous literature.

CONCLUSIONS

Our study brings to light the importance of the proper preparation of hemolyzed samples for the interference quantification in such a particular case as sodium ion. The negative effect by dilution seen in this case makes the protocols including water-dilution steps unsuitable for sodium ion studies, and other more appropriate strategies need to be followed. The establishment of a valid and concentration-independent hemolysis cut-off value will lead to more reliable laboratory results for sodium ion results.

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