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Effect of Hemolysis on Routine Biochemical Parameters

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Abstract

Backgrounds: Hemolysis is still the most common reason for rejecting samples, while reobtaining a new sample is an important problem. The aim of this study was to investigate the effects of hemolysis in different hemolysis levels for mostly used biochemical parameters to prevent unnecessary rejections.

Materials and Methods: The present study was conducted at SMS Medical College & Hospital, Jaipur, India. Nine aliquots, prepared by serial dilutions of homologous hemolyzed samples collected from 20 healthy adult volunteers and containing a final concentration of serum hemoglobin ranging from 0 to 20.6 g/L. Samples were tested for 15 routine biochemical parameters on Fully Automated Analyzer Beckman AU 680 by using standard protocol of commercially available kits.

Results: Hemolysis interference appeared to be approximately linearly dependent on the final concentration of blood-cell lysate in the specimen. This generated a consistent trend towards overestimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, creatine kinase (CK), lactate dehydrogenase (LDH), phosphorus, potassium and urea, whereas mean values of albumin, alkaline phosphatase (ALP), total bilirubin, glucose and sodium were substantially decreased.

Conclusions: To avoid preanalytical visual inspection for hemolysis detection, improper sample rejection, and/or rerun because of hemolysis, it is recommended in this study that, routine determination of plasma or serum free hemoglobin concentrations is important.

Keywords: Hemolysis, Biochemical Parameters, Preanalytical, Free hemoglobin.

Introduction

Hemolysis is one of the most common causes of sub-optimal specimens leading to laboratory error (1). Hemolytic specimens are a frequent occurrence in clinical laboratories, and prevalence can be as high as 3.3% of all of the routine samples, accounting for up to 40%–70% of all unsuitable specimens identified, nearly five times higher than other causes, such as insufficient, incorrect and clotted samples (2). Because some of the constituents of red blood cells (notably potassium and lactate dehydrogenase) are present in the cells at significantly higher concentrations than in serum or plasma, a small amount of hemolysis can lead to significant error if this is not detected (1). Visible hemolysis, usually defined as extracellular hemoglobin concentrations above 0.3 g/L (4.65mol/L), confers a detectable pink to red hue to serum or plasma and is clearly visible in specimens containing as low as 0.5% hemolysate (3). Hemolysis may occur either in vivo or in vitro and is a most undesirable condition that influences the accuracy and reliability of laboratory testing (4). Along with preanalytical causes, in vivo blood cell lysis can originate from hereditary, acquired and iatrogenic conditions, such as autoimmune hemolytic anemia, severe infections, intravascular disseminated coagulation and transfusion reactions (5).

Problems due to troublesome specimen collection or handling, such as wet alcohol transfer from the skin into the blood specimen, small gauge needles (usually smaller than 21 G), difficulty to locate easy venous access, small or fragile veins (alternative sites to the antecubital area, such as hand veins are fragile and easily traumatised), unsatisfactory attempts, vein missing, partial obstruction of catheters and other collection devices, application of excessive negative pressure to the blood in the syringe, underfilling of the tube (excessive concentrations of additives, especially EDTA, can cause rupture of the erythrocytes cell membrane), excessive shaking or mixing of the blood after collection, exposure to excessively hot or cold temperature, centrifugation at a too high speed for a prolonged period of time and centrifugation of partially coagulated specimens from patients on anticoagulants, frequently compromise the integrity of blood and vascular cells, causing leakage of intracellular components and producing significant biological and analytical interference (6-12). Additional causes of in vitro haemolysis are delayed separation of the specimens, incomplete formation of the separator barrier integrity (some erythrocytes can move into serum or plasma) and re-centrifugation (re-spin) of tubes with gel separators (the gel barrier may open and allow any supernatant, which has been in contact with erythrocytes, to mix with the supernatant previously above the separator) (9). When collecting capillary blood, the use of a manual lancet, where the depth of the incision is not controlled, and the excessive squeezing, which induce extreme hydraulic pressure in the capillaries, could also cause haemolysis (9). Finally, in vitro haemolysis has been reported by the use of some innovative pneumatic tube systems (plain serum samples without a gel barrier might be more susceptible to haemolysis than the other sample types) (13). Although rarely, usually less than 2% of all the specimens with detectable haemolysis, haemolytic specimens may also be due to in vivo haemolysis (4). This clinical condition may have at more than 50 causes, including hereditary, acquired and iatrogenic conditions, such as autoimmune haemolytic anaemia and other haemoglobinopathies, drugs, severe infections, intravascular disseminated coagulation, transfusion reactions, heart valves (5) and

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HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome (14). Typically, in vivo haemolysis does not depend on the technique of the healthcare provider and it is thus virtually unavoidable and potentially insurmountable (5). Hemolysis can significantly influence the reliability of a variety of tests for a multitude of biological and analytical reasons. Therefore hemolysis is inherently a challenging problem in laboratory testing, since it may not be evident until whole blood specimen centrifugation has been performed, exposing the serum or plasma to specific scrutiny (15). Regardless of the underlying causes, the potential rejection upon receipt of hemolysed specimens can lead to inconvenience and delay in clinical decision making with substantial implications for the care of patients (15). Although there are comprehensive data on this issue, both from the current literature and from the manufacturers' reagent package inserts, the best solution is still to redefine such limits locally, since many inserts contain limited information on interferences and often only on what concentration of material would interfere with the assay and with no information on what concentrations of analyte were tested. This can be accomplished most reliably by spiking plasma or serum samples with serial dilutions of free hemoglobin, performing the analysis and then identifying the cut offs (i.e., free hemoglobin value). Most of the hemolysed samples are being rejected on pre-analysis stage according to the visual detection of hemolysis, even if the requested tests may not be interfered with hemolysis. Besides, according to the reports, visual assessment of sample hemolysis showed little agreement with the actual concentration of hemoglobin interference (16).

Although laboratory testing of hemolyzed specimens has traditionally been discouraged to avoid unreliable results of some biochemical parameters, there are no definitive guidelines or recommendations on this topic and clinical laboratories usually follow arbitrary or individual procedures to handle results for unsuitable samples due to blood cell lysis. Since the knowledge of possible effects is important for correct interpretation of the results, the aim of the present work was to evaluate the effects of hemolysis interferences in different levels on routine commonly used biochemistry tests. Results of this study may help to prevent unnecessary rejections of samples.

Materials and Methods

The study was conducted on 20 normal healthy subjects of Jaipur, Rajasthan (India) population. Care was taken to ensure that all the normal healthy subjects included in this study are Symptomless for any disease, Free from any abnormality on routine examination, Non-smoker, Non-alcoholic, Not taking Medications. Physical Examination included age, sex, personal history regarding occupation, socio-economic status; habits like food, smoking, alcohol intake, tobacco chewing and drugs of all subjects were carefully recorded. Samples were collected from healthy adult volunteers ranging in age about 25-50 years and informed consent was obtained from each participant. The volunteers were instructed to fast overnight (until blood collection was completed) and to avoid vigorous exercise upon walking. The blood samples of each subject were collected by venepuncture from the anticubital vein after an overnight fast of 10-12 hours. In the morning of the first day of the evaluation, 3.5ml of blood from 20 healthy adult volunteers was separately collected into two 6.0 ml plain serum tubes containing no additives. The first specimen (sample 1) was immediately stored at -70°C whereas the second specimen (sample 2) was

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allowed to clot at room temperature for 60 minutes and centrifuged at 1500xg for 10 minutes using REMI Centrifuge machine. Serum was separated and stored at -70°C. In the morning of the second day of the evaluation, blood from each of the same 20 volunteers was collected into four additional 6.0 ml plain serum tubes. Samples were pooled (sample 3) and finally divided into aliquots of 2 ml. Samples 1 and 2 were thawed; nine serial dilutions of free serum hemoglobin were prepared by mixing scalar aliquots of sample 1 and 2. Then 200 µl of each of these dilutions was added to 2 ml aliquots of blood collected on day 2 (sample 3) to achieve free serum hemoglobin concentrations in the specimens as 0 g/L, 0.16 g/L, 0.30 g/L, 0.60 g/L, 1.30 g/L, 2.60 g/L, 5.10 g/L, 10.30 g/L and 20.6 g/L, thus being almost representative of the hemolysis degree we randomly observe in samples sent to our laboratory. Hemolysis was assayed by measuring the concentration of free serum hemoglobin by the reference cyanmethemoglobin method on the UV-1700 spectrophotometer (17). Blood was then allowed to clot for 60 minutes at room temperature; centrifuged at 1500xg for 10 minutes; serum was separated and immediately analyzed for fifteen routine biochemical parameters which includes Glucose, Urea, Creatinine, Aspartate transaminase, Alanine transaminase, Alkaline Phosphatase, Lactate dehydrogenase, Creatine Kinase, Acid phosphatase, Total Bilirubin, Total Cholesterol, Triglyceride, Total Protein, Albumin, Uric Acid, Calcium, Phosphorus, Sodium, Potassium on Fully Automated Analyzer Beckman AU 680 by using standard protocol of commercially available kits.

Statistical Analysis

To compare the concentrations of the hemolyzed samples with nonhemolyzed samples, bias percentage was calculated by the formula:

$[(CX – C1)/ C1)] \times 100$.

C1: concentration of non hemolyzed sample,

CX: concentration of hemolyzed sample.

All analyses were performed using Statistical Package for Social Sciences statistical package (SPSS, version 15.0 for Windows XP) (18). The clinical significance was calculated as bias % with p value < 0.05. Interference effects were checked by Wilcoxon signed rank test for paired data when concentrations were compared with baseline. The criterion for the presence of a bias specific for that analyte was defined according to the desirable bias recommended by CLIA'88 guidelines.

Result

Table1: Values of routine biochemical parameters expressed as mean±SD in serum containing increased concentrations of free hemoglobin (n=20)

Analytes	No Lysis	Free Serum Hemoglobin (g/L)							
		0.16	0.30	0.60	1.30	2.60	5.10	10.30	20.60
Glucose (mg/dl)	85.25±3.9	85.26±4.0	85.25±3.9	85.24±3.8	85.23±4.0	85.22±3.8	85.20±3.7	85.14±3.9	85.12±3.8
Urea (mg/dl)	33.43±2.3	33.44±2.2	33.42±2.1	33.43±2.3	33.44±2.2	33.51±2.1	33.56±2.2	33.62±2.4	33.75±2.3
Creatinine (mg/dl)	0.81±0.10	0.82±0.11	0.82±0.11	0.83±0.10	0.84±0.11	0.85±0.10	0.86±0.11	0.86±0.10	0.87±0.09
AST (U/L)	22.62±4.2	23.53±4.3	25.38±4.4	27.28±4.2	31.26±4.3	40.21±5.1	59.84±6.8	95.73±7.2	169.24±8.4
ALT (U/L)	20.58±5.6	20.67±5.7	20.69±5.6	20.71±5.5	20.76±5.7	22.24±5.5	23.01±5.6	26.35±5.7	32.48±5.5
ALP (U/L)	52.24±6.2	52.26±6.3	52.23±6.1	51.34±6.4	50.58±6.2	47.02±5.9	43.79±5.8	36.71±5.7	24.64±5.2
LDH (U/L)	201.2±7.4	208.4±7.3	224.6±7.5	256.8±7.4	294.7±7.5	316.8±7.6	352.5±8.4	424.6±8.7	626.8±9.2

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CK (U/L)	118.5±6.5	119.1±6.6	119.9±6.7	121.1±6.8	122.5±7.1	123.8±7.5	125.6±7.7	142.4±8.1	167.7±8.5
Albumin (g/dl)	4.29±0.40	4.27±0.42	4.25±0.44	4.24±0.43	4.23±0.41	4.21±0.42	4.20±0.40	4.15±0.44	4.11±0.41
Uric Acid (mg/dl)	4.26±0.42	4.19±0.41	4.18±0.43	4.17±0.42	4.12±0.44	4.10±0.43	4.06±0.41	4.02±0.40	3.99±0.43
T. Bilirubin (mg/dl)	0.42±0.13	0.41±0.14	0.40±0.13	0.39±0.10	0.38±0.14	0.38±0.14	0.36±0.12	0.35±0.15	0.34±0.14
Calcium (mmol/L)	2.40±0.09	2.40±0.10	2.40±0.09	2.40±0.10	2.41±0.09	2.41±0.09	2.42±0.10	2.42±0.09	2.40±0.10
Phosphorus(mmol/L)	1.17±0.12	1.17±0.11	1.17±0.11	1.18±0.12	1.18±0.11	1.20±0.11	1.23±0.11	1.29±0.11	1.42±0.12
Sodium (mmol/L)	140.1±1.5	139.6±1.1	139.2±1.0	138.9±0.8	138.7±1.0	138.6±1.2	137.9±1.4	136.5±0.9	133.5±1.0
Potassium(mmol/L)	4.12±0.22	4.15±0.22	4.18±0.23	4.27±0.21	4.34±0.22	4.65±0.24	5.22±0.25	6.36±0.30	8.78±0.42

Table 2: P- values for Changes in Analytes Concentrations with Increasing Hemolysis, % bias of analyte concentrations in comparison to no lysis values and desirable bias.

Analytes	Free Serum Hemoglobin (g/L)								Desirable Bias %
	0.16	0.30	0.60	1.30	2.60	5.10	10.30	20.60	
Glucose (mg/dl)	0.9937 0.01	1.0000 0.0	0.9935 -0.01	0.9873 -0.02	0.9805 -0.03	0.9670 -0.05	0.9294 -0.12	0.9155 -0.15	±2.2
Urea (mg/dl)	0.9889 0.02	0.9886 -0.02	1.0000 0.0	0.9889 0.02	0.9092 0.23	0.8560 0.38	0.7996 0.56	0.6625 0.95	±5.5
Creatinine (mg/dl)	0.7652 1.23	0.7652 1.23	0.5309 2.46	0.3725 3.70	0.2136 4.93	0.1408 6.17	0.1221 6.17	0.0533 7.40	±3.4
AST (U/L)	0.5025 4.02	0.0495 12.20	0.0012 20.60	<0.001 38.10	<0.001 77.76	<0.001 164.50	<0.001 323.20	<0.001 647.60	±5.4
ALT (U/L)	0.9601 0.43	0.9508 0.53	0.9413 0.63	0.9203 0.87	0.3502 8.06	0.1780 11.80	0.0026 28.0	<0.001 57.82	±12.0
ALP (U/L)	0.9920 0.03	0.9959 -0.01	0.6541 -1.72	0.4025 -3.17	0.0096 -9.99	0.0016 -16.17	<0.001 -29.72	<0.001 -52.83	±6.4
LDH (U/L)	0.0037 3.57	<0.001 11.6	<0.001 27.6	<0.001 46.4	<0.001 57.4	<0.001 75.1	<0.001 111.0	<0.001 211.3	±4.3
CK (U/L)	0.7736 0.50	0.5065 1.18	0.2240 2.19	0.0709 3.37	0.0220 4.47	0.0032 5.99	<0.001 20.10	<0.001 41.51	±11.5
Albumin (g/dl)	0.8783 -0.46	0.7652 -0.93	0.7055 -1.16	0.6421 -1.39	0.5410 -1.86	0.4811 -2.09	0.2990 -3.26	0.1680 -4.19	±1.3
Uric Acid (mg/dl)	0.5969 -1.64	0.5552 -1.87	0.5021 -2.11	0.3098 -3.28	0.2413 -3.75	0.1358 -4.69	0.0720 -5.63	0.0517 -6.33	±4.8
Total Bilirubin (mg/dl)	0.8162 -2.38	0.6294 -4.76	0.4184 -7.14	0.3550 -9.52	0.3550 -9.52	0.1376 -14.28	0.1231 -16.66	0.0688 -19.04	±10.0
Calcium (mmol/L)	1.0000 0.0	1.0000 0.0	1.0000 0.0	0.7273 0.41	0.7273 0.41	0.5102 0.83	0.4865 0.83	1.0000 0.0	±0.8
Phosphorus(mmol/L)	1.0000 0.0	1.0000 0.0	0.7936 0.85	0.7850 0.85	0.4150 2.56	0.1075 5.12	0.0021 10.25	<0.001 21.3	±3.2
Sodium (mmol/L)	0.2368 -0.35	0.0315 -0.64	0.0031 -0.85	0.0013 -0.99	0.0012 -1.07	<0.001 -1.57	<0.001 -2.56	<0.001 -4.71	±0.3
Potassium (mmol/L)	0.6687 0.72	0.4045 1.45	0.0335 3.64	0.0031 5.33	<0.001 12.86	<0.001 26.69	<0.001 54.36	<0.001 113.1	±1.8

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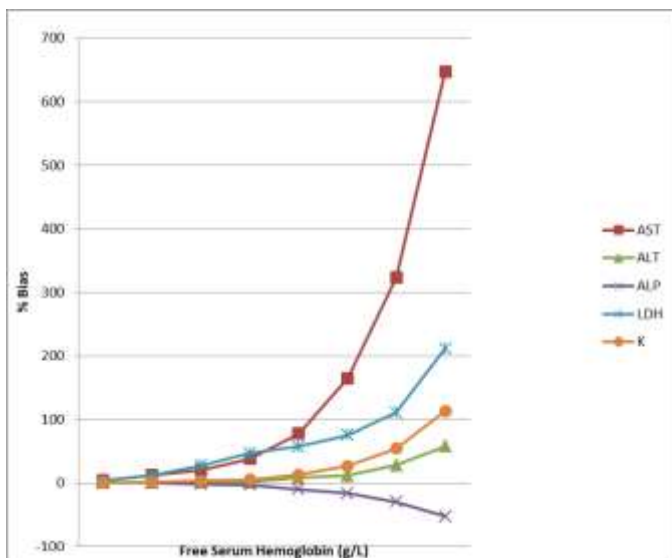

Figure 1: Interferogram for free serum hemoglobin and measured parameters: AST, ALT, ALP LDH and Potassium

The results of the present investigation on hemolyzed specimens are presented in Tables 1 and 2 and shown in Figure1. Table1 & 2 summarizes the influence of hemolysis on routine biochemistry testing. Differences are given as mean ±SD, P- values and percentage relative bias from the baseline specimens (no lysis). As expected, LDH, ALT, AST and potassium values showed significant increases approximately linearly dependent on the free Hb concentrations in hemolyzed plasma. There was a decrease in total bilirubin and ALP concentrations (Figure 1). Hemolysis interference, expressed as mean absolute and percentage relative bias, appeared to be approximately linearly dependent on the final concentration of blood cells lysate in the specimen. As expected, the addition of blood cell lysate generated a consistent and dose-dependent trend towards overestimation of ALT, AST, creatinine, CK, LDH, phosphorus and potassium whereas mean values of albumin, ALP and total bilirubin were substantially decreased when compared with the baseline specimens (no lysate). Statistically significant differences in samples containing 0.6 g/L of serum hemoglobin or less could already be observed for AST, ALT, LDH, CK and potassium. Clinically meaningful variations, as reflected by deviation from the current analytical quality specifications for desirable bias (19), were observed for AST, ALT, ALP, LDH and potassium in samples with mild or almost undetectable hemolysis by visual inspection (0.6 g/L of serum hemoglobin) (Table 2).

Discussion

Hemolysis is the most frequent reason for specimen rejection, as indicated by the College of American Pathologists (CAP) Chemistry Specimen Acceptance Q-Probes Study, as it influences the reliability of laboratory tests for both biological and analytical reasons (20). Leakage of intracellular analytes in plasma might produce falsely elevated measurable concentrations or dilutional effects increase the optical absorbance or change the blank value, producing method- and analyte concentration-dependent spectrophotometric interference in common laboratory assays. Unfortunately, clinically meaningful variations of some biochemical and coagulation tests were observed in specimens displaying hemolysis that is mild or almost undetectable by visual inspection (serum hemoglobin -0.3 g/L) (1).

The interference of in vitro hemolysis on laboratory testing might be caused by: (i) leakage of hemoglobin and other intracellular components into the surrounding fluid, which induces false elevations of some analytes or dilution effects; (ii) chemical interference by free hemoglobin in the analytical reaction; and (iii) method- and analyte concentration-dependent spectrophotometric interference due to an increase in the optical absorbance or a change in the blank value, especially for laboratory measurements at 415, 540 and 570 nm, where hemoglobin shows strong absorbance (21).

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Although the interference seems to be related to the degree of lysis and the specificity of the method being used, it has been reported that several laboratory results can be seriously influenced, especially those for potassium, sodium calcium, magnesium, bilirubin, haptoglobin, total protein, aldolase, amylase, LDH, AST, ALT, phosphorus, alkaline phosphatase, acid phosphatase, GGT, folate and iron (22).

In the present study, influence of hemolysis was examined on 15 routine biochemical parameters. Nine aliquots were prepared by serial dilutions of homologous hemolyzed samples collected from 20 different subjects and containing a final concentration of serum hemoglobin ranging from 0 to 20.6 g/L (Table 1,2).

Statistically significant increase in samples containing 0.16 g/L ($P<0.01$), 0.30 g/L ($P<0.001$), 0.60 g/L ($P<0.001$), 1.30 g/L ($P<0.001$), 2.60 g/L ($P<0.001$), 5.10 g/L ($P<0.001$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin could already be observed for LDH. AST concentration was significantly high in samples containing 0.30 g/L ($P<0.05$), 0.60 g/L ($P<0.01$), 1.30 g/L ($P<0.001$), 2.60 g/L ($P<0.001$), 5.10 g/L($P<0.001$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin; Sodium concentration was significantly low in samples containing 0.30 g/L ($P<0.05$), 0.60 g/L ($P<0.01$), 1.30 g/L ($P<0.01$), 2.60 g/L ($P<0.01$), 5.10 g/L($P<0.001$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin and Potassium concentration was significantly high in samples containing 0.60 g/L ($P<0.05$), 1.30 g/L ($P<0.01$), 2.60 g/L ($P<0.001$), 5.10 g/L($P<0.001$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin (Table 1,2; Figure 1) as compared to reference specimen (no lysis).

ALT concentration was significantly high in samples containing 10.30 g/L ($P<0.01$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin; Alkaline phosphatase concentration was significantly low in samples containing 2.60 g/L ($P<0.01$), 5.10 g/L ($P<0.01$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin; Creatine kinase concentration was significantly high in samples containing 2.60 g/L ($P<0.05$), 5.10 g/L($P<0.01$), 10.30 g/L ($P<0.001$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin and Phosphorus concentration was significantly high in samples containing 10.30 g/L ($P<0.01$) and 20.6 g/L ($P<0.001$) of free serum hemoglobin as compared to the baseline specimen. Albumin, total bilirubin, calcium, glucose, urea creatinine and uric acid concentrations were not significantly different, even in specimens containing up to 20.6 g/L of free serum hemoglobin (Table 1,2; Figure 1).

The activity of AST in erythrocytes is 40 times higher than in plasma. In patients with AST activities in the reference interval, hemolysis with hemoglobin values of 150 mg/dL causes an elevated AST activity (6). As for ALT, AST, LDH, magnesium, phosphorus and potassium, the interference mechanism has previously been characterized and is attributed to large differences between intracellular and extracellular concentrations for these analytes. Lower values for glucose, sodium and chloride are likely caused by dilution effects, whereas hemolysis interference in the CK assay has been attributed to intracellular adenylate kinase, which is not wholly inhibited under operating conditions. For ALP, iron, lipase and GGT, hemolysis interference is likely caused by spectral overlap and by a chemical reaction between hemolysate and reaction components (24).

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Nowadays serum hemolysis index is a popular solution for interference detection preanalytically. Manufacturers give the list of test-specific serum indices for hemolysis, lipemia and bilirubin interferences. This can help laboratory staff to beware of interference, study or reject the sample, and add comments to the results. But the standardization problem of the various analytical systems and different decision thresholds for various serum indices requires more effort (25).

Summary and Conclusion

Since hemolysis can affect the integrity of the specimen and the reliability of laboratory results, standardized reporting of the occurrence of hemolysis may support the improvement of the quality and efficiency of the preanalytical laboratory process. Thus whenever a hemolysed sample is received in the laboratory it should not be rejected, free hemoglobin levels should be quantified first. We therefore recommend routine free Hb level determination in serum or plasma, or any other automated detection of the degree of hemolysis. Only for those analyses which are affected by the estimated degree of hemolysis, new samples have to be requested. Although time consuming, this procedure is highly useful, since it may help to avoid unnecessary rerun the samples and reduce the costs.

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