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Original Research

GESDAV

Interference of hemolysis in the estimation of plasma aspartate aminotransferase, potassium and phosphate

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ABSTRACT

Objective: The objective was to analyze the effect of hemolysis on estimation of biochemical constituents in the plasma. **Materials and Methods:** Twenty different pooled plasma samples were collected from 80 apparently healthy volunteers at Alnaw Hospital and Khartoum teaching hospital and research laboratory unit. Hemolysate was prepared by freezing at −20°C overnight and serial dilution of hemolysate was prepared (0.125, 0.25, 0.5, 1, 2, 4, and 8 g/dl). Each pooled plasma samples was dispensed into 8 different tubes (900 μl in each tube) to which 100 μl of different concentration of the hemolysate were added in each of the tubes. A baseline control was prepared with 100 μl of distilled water. Plasma aspartate aminotransferase (AST), potassium and phosphate were measured in each of the tubes. Plasma level of AST and phosphate were measured using Hitachi 902 Analyzer (USA for Hitachi 920 analyzer), whereas potassium was measured using ion-selective electrode. **Result:** The result showed that plasma AST, potassium, and phosphate were significantly raised at hemoglobin (Hb) concentration of 0.4 and 0.8 g/dl ($P < 0.0l$), whereas plasma potassium and AST was significantly increased at Hb concentration of 0.1 and 0.2 g/dl ($P < 0.01$). Further at 0.05 g/dl Hb concentration, only plasma potassium was significantly increased ($P < 0.05$) but not others. **Conclusion:** There will definitely be a great influence of hemolysis on the estimation biochemical parameters in the plasma giving false-positive values.

KEY WORDS: Aspartate aminotransferase, false values, hemolysate, phosphate, potassium

INTRODUCTION

Hemolysis is an important interference factor that must be considered when making laboratory measurements. Its influence should not be ignored. Because of the automation of many analytical processes, including increased automation of the pre-analytical phase, screening test material for hemolysis is often lacking. In particular, samples collected for complete blood testing in non-laboratory settings have to be transported over longer distances because of the increasing consolidation of laboratories. As a result, there is an increased risk of hemolysis during storage and/or transportation. Even if hemolysis is not visually detectable, a discharge of intracellular constituents into the plasma/serum can have occurred. If the tests are on patients with the hemolytic syndrome (*in vivo* hemolysis), then differentiating it from *in vitro* hemolysis, mostly resulting from inappropriate specimen collection, is rarely possible. Consequently, analytical results are often false, fluctuating between either too high or too low, or giving unexpected pathological findings, for such measurements as potassium, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), acid phosphatase, and/or neuron-specific enolase.

Hemolysis is the release of intracellular components from erythrocytes, thrombocytes and leukocytes into the extracellular fluid, i.e., the plasma or serum [1]. Hemolysis is visible as red coloration of plasma or serum after centrifugation of the sample. A report in the literature on the concentration of free hemoglobin (Hb), which is visible as red coloration in the plasma or serum varies between 100 and 300 mg/l. Hemolysis can lead to changes of a specific parameter in the test material. It is called a biological influence factor if the release of the blood-cell constituents took place *in vivo*. *In vitro* hemolysis is an interference factor if it occurs after specimen collection and changes the results of the analytical process. Thrombocytolysis and granulocytolysis can also influence test results without visual hemolysis. On examination of the coagulation process, it is evident that thrombocytolysis is responsible for the higher concentration of a number of intracellular components in serum compared to the plasma. An intravascular destruction of leukocytes can lead to increased lysozyme levels in myeloid and monocytic leukemia's [l].

In vivo hemolysis is caused by antibodies, biochemically through medications, by toxic substances, through hereditary

factors (e.g., hemoglobinopathies), through enzyme defects or by infections (e.g. malaria). When suspecting *in vivo* hemolysis the plasma should be checked to exclude the possibility of additional *in vitro* hemolysis caused by the coagulation process.

In vivo hemolysis may be distinguished from *in vitro* hemolysis by comparing a hemolytic sample of a patient with other samples from the same patient, arriving at the same time. Free Hb *in vivo* rapidly binds to haptoglobin and the complex is eliminated from the circulating blood (as in hemolytic anemia). Consequently, haptoglobin is reduced during intravasal hemolytic process. The measurement of low concentration of haptoglobin thus permits an imperative assessment of hemolysis (exceptions are inborn haptoglobin deficiency and newborn children). Likewise, the measurement of hemopexin and/or methemoglobin-albumin was used to characterize *in vivo* hemolysis [2]. A rise in concentration of indirect bilirubin and reticulocyte counts is a typical sign of *in vivo* hemolysis, which in turn leads to increased erythropoiesis. Other consequences of *in vivo* hemolysis, such as a change in the LD isoenzyme pattern, seem less suitable for the identification of hemolysis because of their low diagnostic sensitivity and specificity. *In vivo*-hemolysis parallel the increase in Hb (red coloration of plasma/serum) and LD, but no parallel increase in potassium. No red coloration of plasma/serum, but decrease in haptoglobin and potential increase in LD, indirect bilirubin and/or reticulocyte-index, respectively (*in vivo*).

Subsequent to *in vitro* hemolysis all constituents of erythrocytes, including potassium concentration, LDH and aspartate aminotransferase activities, increase in addition to the concentration of free Hb in plasma or serum [3]. In contrast, haptoglobin concentration in plasma/serum of hemolytic samples remains unchanged. Certain immunological methods differ in their ability to distinguish Hb/haptoglobin complexes from free haptoglobin [2]. *In vitro*-hemolysis parallel the increase of Hb (red coloration of plasma/serum), potassium, LD and AST, respectively. However, haptoglobin and reticulocyte-index remains normal. Unforeseen increase in potassium, but no red coloration of plasma/serum, LD in the reference range, i.e. if whole blood is stored for several days (*in-vitro*). Serum without red coloration, but there is the increase in LD, potassium and acid phosphatase. In plasma, there is no increase of these parameters (i.e., has been noticed in thrombocytosis) (*in vitro*).

The intracellular concentration of most of the constituents is 10 times higher as compared with the extracellular fluid. Thus, the extracellular concentration of many constituents (e.g., potassium, LD, aspartate aminotransferase) will increase in plasma/serum upon hemolysis. Blood cell constituents can directly or indirectly interfere in the measurement of analytes. Adenylate kinase released from erythrocytes causes an increase of creatine kinase (CK) and CK-MB subunit (CK-MB) activity especially when inhibitors of adenylate kinase in the assay mixture are inadequate [4]. In contrast, adenylate kinase does not affect the immunochemical quantification of CK-MB. Pseudo-peroxidase activity of free Hb interferes in the bilirubin procedure of Jendrassik and Groof by inhibiting the diazonium color formation leading to false low result [5].

The effect of hemolysis on various analytes measured in clinical chemistry has been thoroughly investigated [6-8]. Most often, the color of Hb increases the absorption at a respective wavelength or changes the blank value. An apparent increase or decrease of a result by Hb is therefore method-and analyte concentration-dependent. Likewise, the changes caused by therapeutic Hb derivatives are primarily due to optical interference [9-11].

MATERIALS AND METHODS

This study was based on nonprobability volunteers sampling technique. 2.5 ml of venous blood was collected from each volunteer, after sterilization of the arm by 70% alcohol.

Hemolysate was prepared as follows:
1. A volume of 5 ml of whole blood sample (with normal profile) was obtained on lithium heparin, (in gel free tubes) and centrifuged
2. Plasma was removed, and equal volume of normal saline was added
3. The cells was re-suspended and centrifuged
4. The Steps 2 and 3 were repeated 3 times
5. Distilled water was added, and then the solution was stored at −20°C overnight
6. The frozen solution was allowed to thaw, then mixed, and centrifuged.

Supernatant (hemolysate) was transferred to a clean tube and Hb was measured by using mythic hematology analyzer (Diamond Diagnostics - USA for mythic hematology analyzer). Eight serial dilutions of hemolysate were prepared to obtain Hb concentration of 0.125, 0.25, 0.5, 1, 2, 4, and 8 g/dl.

Spike sample was prepared as follows:
1. Twenty different pooled plasma samples were collected
2. 100 µl of distilled water was added to 900 µl of each of the pooled plasma samples as baseline
3. 100 µl of each of the serial concentration of Hb was added to 900 µl of each pooled plasma sample that is added concentration was 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 g/dl
4. AST, phosphate and K + were measured 2 times in each spiked samples and the means was calculated.

Ethical Consideration

The objectives of the study were explained to all individual participating in this study. Permission of this study was obtained from local authorities in the area of study.

Roche Diagnostic, Hitachi 902 analyzer was used to measure and report the plasma level of AST activity and phosphate. This analyzer is fully automated, computerized and include: Photometric measuring system, analytical processing unit, touch

screen, and printer. Roche Diagnostic, Hitachi 902 was used to measure AST and phosphate in the plasma samples. Potassium was estimated by ion-selective electrode.

Statistical Analysis

The data collected in this study were analyzed using SPSS computer analysis program. Statistical significance was tested using analysis of variance.

RESULTS

Plasma Aspartate Transaminase

As described in Table 1 and shown in Figure 1, the plasma AST activity was 28 U/l at base line and 29 U/l after adding 0.0125 g/dl of hemolysate, so the difference between the two readings is insignificant ($P > 0.05$).

At the spike sample with 0.025, 0.05 and 0.1 g/dl Hb concentration mean of plasma AST activity was 30, 32, and 36, U/l, respectively, which is statistically insignificant when compared with the mean of baseline ($P > 0.05$).

At the spike sample with 0.2, 0.4 and 0.8 g/dl Hb concentration mean of plasma AST activity was 44, 59, and 97 U/l, respectively, which is statistically significant when compared with the mean of baseline ($P < 0.01$).

Table 1: Comparison of mean plasma AST between baseline and spike samples with different concentrations of Hb

Variable	Plasma AST (U/L±SD)	*P* value
Base line	28±7.84	–
Spike 0.0125	29±7.81	0.999
Spike 0.025	30±7.77	0.929
Spike 0.05	32±7.81	0.438
Spike 0.1	36±7.81	0.010*
Spike 0.2	44±7.78	0.000**
Spike 0.4	59±7.85	0.000**
Spike 0.8	97±7.75	0.000**

*$P<0.05$, **$P<0.01$, The values are the mean of 20 measurements, Dunnett *t*-tests treat one group as a control (base line) to compare all other groups against it, ANOVA test was used for comparison, AST: Aspartate amino transferase, Hb: Hemoglobin

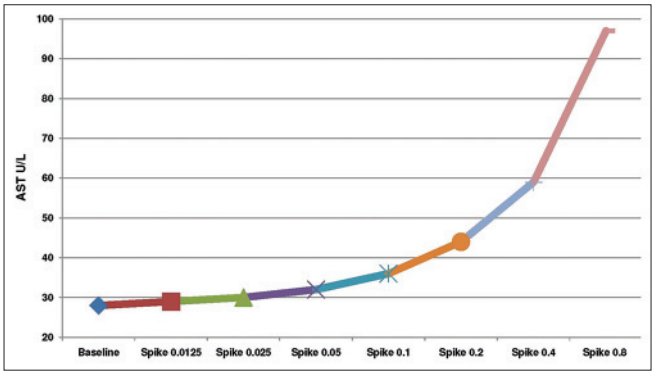

Figure 1: Effect of hemolysis on mean plasma aspartate amino transferase between baseline and spike samples with different concentrations of hemoglobin

Plasma Potassium

Table 2 and Figure 2 show strong positive correlation between potassium and Hb concentration. The plasma potassium concentration was 3.46 mmol/l, at baseline and 3.53 mmol/l, after adding 0.0125 g/dl hemolysate. The difference between the two readings is insignificant ($P > 0.05$). At the spike sample with 0.025 g/dl Hb concentration mean of plasma potassium concentration was 3.57 mmol/l, which is statistically insignificant when compared with the mean of base line ($P > 0.05$).

The spike sample with 0.05, 0.1, 0.2, 0.4, and 0.8 g/dl Hb concentration the mean plasma potassium was 3.66, 4.84, 4.17, 4.48, and 6.08 mmol/l, respectively, which is statistically significant when compared with the mean of baseline [Table 1].

Plasma Phosphate

As described in Table 3 and Figure 3, the plasma phosphate concentration measured 3.55 mg/dl at baseline and 3.58 mg/dl after adding 0.0125 g/dl hemolysate, and the difference between the two readings is insignificant ($P > 0.05$).

At the spike sample with 0.025, 0.05, 0.1, and 0.2, g/dl Hb concentration the mean plasma phosphate concentration was 3.62, 3.65, 3.67, and 3.88 mg/dl, respectively, which is statistically insignificant when compared with the mean of baseline ($P > 0.05$).

Table 2: Comparison of mean plasma potassium between baseline and spike samples with different concentrations of Hb

Variable	Plasma potassium (mmol/l±SD)	*P* value
Base line	3.46±0.18	–
Spike 0.0125	3.53±0.16	0.905
Spike 0.025	3.57±0.17	0.520
Spike 0.05	3.66±0.15	0.030*
Spike 0.1	3.84±0.15	0.000**
Spike 0.2	4.17±0.16	0.000**
Spike 0.4	4.84±0.21	0.000**
Spike 0.8	6.08±0.44	0.000**

*$P<0.05$, **$P<0.01$, The values are the mean of 20 measurements, Dunnett *t*-tests treat one group as a control (base line) to compare all other groups against it, ANOVA test was used for comparison. Hb: Hemoglobin

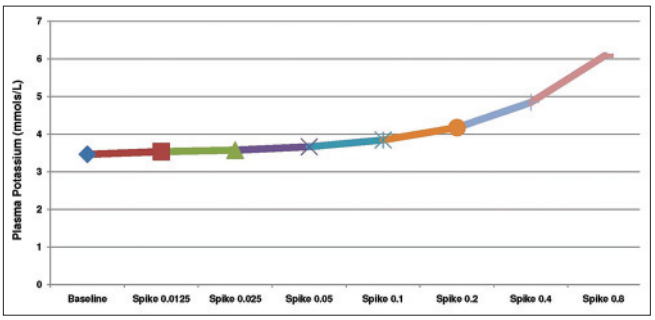

Figure 2: Effect of hemolysis on mean plasma Potassium between baseline and spike samples with different concentrations of hemoglobin

At the spike sample with 0.4 and 0.08 g/dl Hb concentration mean plasma phosphate concentration was 4.3 and 5.1 mg/dl, which is statistically significant when compared with the mean of baseline ($P < 0.01$).

As shown in Table 4 and Figure 4, the interference of Hb was proportional to the plasma Hb concentration which began as low as 0.05 g/dl. There was positive interference on AST, potassium and phosphate measurement. At plasma Hb concentration of 0.8 g/dl, the values of AST, potassium and phosphate were increased by 246%, 76% and 44%, respectively ($P < 0.01$).

DISCUSSION

In the present study, hemolysis defined as the release of intracellular components from erythrocytes, thrombocytes and leukocytes into the extracellular fluid that is the plasma or serum. Hemolysis is visible as red coloration of plasma or serum after centrifugation of the sample. Hemolysis can lead to changes of specific parameters in the tests material. It is called as biological influence factor if the release of the blood-cell constituents took place *in vivo*. *In vitro* hemolysis is an interference factor if it occurs after specimen collection and changes the results of the analytical process. Thrombocytolysis and granulocytolysis can also influence test results without visual hemolysis. On examination of the coagulation process, it is evident that thrombocytolysis is responsible for the higher concentration of a number of intracellular components in serum compared to the plasma [1].

Potassium is the major intracellular cation in the body, with concentration 20 times greater inside the cell than outside. Many cellular functions require that the body maintain a low ECF concentration of potassium [12]. As a result, only 2% of the body total potassium circulates in the plasma.

Phosphate is an essential component of cellular membrane lipid bilayer (phospholipids) and intracellular compounds like nucleic acids and nucleoprotein, and most intracellular phosphates exist as organic phosphate in creatine phosphate, adenosine triphosphates and 2-3 diphosphoglycerate. The total body phosphorus is around 700 g (23,000 mmol) and is distributed mainly in the bones (80%), viscera (10.9%), skeletal muscle (9%), and only 0.1% is in the extracellular space [13].

AST is widely distributed in human tissue. The highest concentration found in cardiac tissue, liver and skeletal muscle, with smaller amounts found in the kidney, pancreas and erythrocytes [14] the activity of AST in erythrocytes is ×40 higher than in plasma.

Table 3: Comparison of mean plasma phosphate between base line and spike samples with different concentrations of Hb

Variable	Plasma phosphate (mg/dl±SD)	*P* value
Base line	3.55±0.41	–
Spike 0.0125	3.58±0.42	1.000
Spike 0.025	3.62±0.42	0.997
Spike 0.05	3.65±0.41	0.989
Spike 0.1	3.67±0.42	0.958
Spike 0.2	3.88±0.49	0.159
Spike 0.4	4.30±0.51	<0.001*
Spike 0.8	5.10±0.65	<0.001*

*$P<0.001$, The values are the mean of 20 measurements, Dunnett *t*-tests treat one group as a control (base line) to compare all other groups against it, ANOVA test was used for comparison. Hb: Hemoglobin

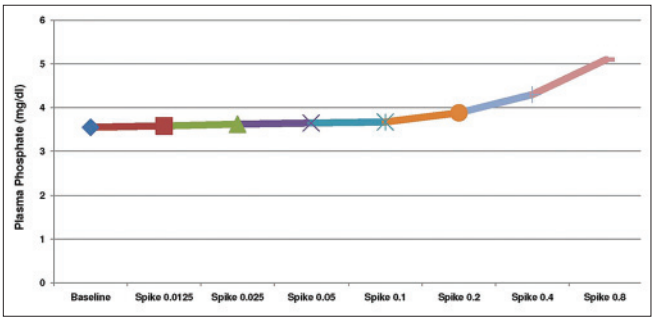

Figure 3: Effect of hemolysis on mean plasma Phosphate between baseline and spike samples with different concentrations of hemoglobin

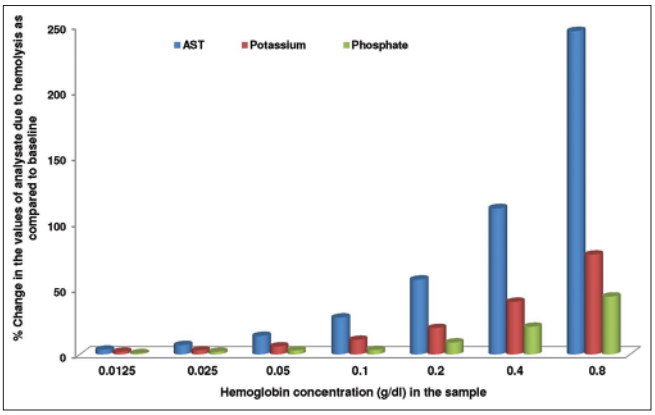

Figure 4: Effect of hemolysis on percent change in some analysates between baseline and spike samples with different concentrations of hemoglobin

Table 4: Effect of hemolysis in the measurement of plasma concentration of some analytes

Analytes	Hb concentration (g/dl)							
	0	0.0125	0.025	0.05	0.1	0.2	0.4	0.8
AST U/l	28	29	30	32	36*	44**	59**	97**
% Change from baseline value	–	3.5	7	14	28	57	111	246
Potassium mmol/l	3.46	3.53	3.57	3.66*	3.84**	4.17**	4.84**	6.08**
% Change from baseline value	–	2	3	6	11	20	40	76
Phosphate mg/dL	3.55	3.58	3.62	3.65	3.67	3.88	4.30**	5.10**
% Change from baseline value	–	0.8	2	3	3.3	9	21	44

*$P<0.05$, **$P<0.01$. The values are mean of 20 measurements, % Change has been compared with 0 g/dl Hb (base line). Hb: Hemoglobin

The interference of *in vitro* hemolysis on laboratory testing might be caused by: (1) Leakage of Hb and other intracellular components into the surrounding fluid, which induces false elevations of some analytes or dilution effects; (2) chemical interference by free Hb in the analytical reaction (3) 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 Hb shows strong absorbance [5,15,16].

In this study, the increase of plasma levels of AST, potassium and phosphate was proportional to the plasma Hb concentration, which began as low as 0.05 g/dl. There was positive interference on phosphate, potassium, and AST measurement. At a plasma Hb concentration of 0.8 g/dl, the values of phosphate, potassium, and AST were increased by 44%, 76%, and 246%, respectively ($P < 0.01$).

At a plasma Hb concentration of 0.4 g/dl, the values of phosphate, potassium, and AST were increased by 21%, 40%, and 111%, respectively ($P < 0.01$). At a plasma Hb concentration of 0.2 g/dl, the values of potassium and AST were increased by 20% and 57%, respectively ($P < 0.01$). At a plasma Hb concentration of 0.1 g/dl, the values of potassium and AST were increased by 11% ($P < 0.05$) and 28%, respectively ($P < 0.01$). At a plasma Hb concentration of 0.05 g/dl, the value of potassium only was increased by 6%, ($P < 0.05$). Hemolysis causes positive interference in phosphate assays due to inorganic phosphates produced by the action of phosphatases on organic phosphates, both being released from red cells on hemolysis.

The AST activity in red cells is greater 40 times than in plasma; hence, hemolysis results in interference in the assay and measurement of AST. Hemolysis of red blood cells releases large amounts of potassium into the surrounding plasma. The concentration of potassium in red blood cells is approximately ×25 higher than in plasma. The concentration of potassium is increased; even if the *in vitro* hemolysis is not visible through red coloration.

The recent study done by Lippi *et al.* [17], were in agreement, who found the increase degree of hamolysate leads to false increase of AST, potassium and phosphate and attributed to the large difference between intracellular and extracellular concentrations for these analytes.

CONCLUSION

On the basis of the results of this study, it could be concluded that:

1. Plasma AST activity in U/l was significantly increased in spike sample with Hb concentration of 0.1, 0.2, 0.4, and 0.8 g/dl when compare with baseline sample and not significantly at concentration 0.0125, 0.025, and 0.05 g/dl
2. Plasma potassium concentration in mmo1/l was significantly increased in spike sample with Hb concentration of 0.05, 0.1, 0.2, 0.4, and 0.8 g/dl when compared with base line sample and not significantly at concentration 0.0125 and 0.025 g/dl
3. Plasma phosphate concentration in mg/dl was significantly increased in spike sample with Hb concentration of 0.4 and 0.8 g/dl when compared with base line sample, but no significant difference at lower concentrations.

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