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EFFECT OF BLOOD CELL SUBTYPES LYSIS ON ROUTINE BIOCHEMICAL TESTS

EFEKAT HEMOLIZE PODTIPOVA KRVNIH ĆELIJA NA RUTINSKE BIOHEMIJSKE TESTOVE

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Summary

Background: The aim of this study is to establish the contribution of blood cells subtypes on hemolysis.
Methods: Separated blood cell subtype suspensions prepared with blood from 10 volunteers were serially diluted to obtain different concentrations of cell suspensions. The cells were fully lysed and cell hemolysates were added (1:20) to aliquots of serum pool. Thus, seven serum pools with different concentrations of interferent were obtained for each blood cell subtype. Biochemical parameters and serum indices were measured by an autoanalyzer. As cell lysis markers, free hemoglobin was measured by spectrophotometry while myeloperoxidase and β-thromboglobulin were measured by enzyme immunoassay. The percent changes in analyte levels of the serum pools were evaulated by Wilcoxon Signed Rank Test and compared with clinical thresholds defined for each test.
Results: The clinical thresholds were exceeded in lactate dehydrogenase, potassium, aspartate aminotransferase, creatine kinase, magnesium, total protein, total cholesterol, inorganic phosphate, glucose for red blood cells (RBC); lactate dehydrogenase, aspartate aminotransferase, total protein, inorganic phosphate and glucose for platelets (PLT). Free hemoglobin was significantly correlated with RBC ($r=0.999$; $p=0.001$), while myeloperoxidase and β thromboglobulin showed no significant correlation to white blood cells (WBC) and PLT, respectively.
Conclusions: The effect of RBC hemolysis in serum on the routine biochemical tests are clearly established, yet, additional studies are required in order to verify this kind of effects of PLT and WBC hemolysis.

Keywords: hemolysis, interference, biochemical test, free hemoglobin, myeloperoxidase

Kratak sadržaj

Uvod: Cilj ovog proučavanja je da se utvrdi uticaj podtipova krvnih ćelija na hemolizu.
Metode: Suspenzija izdvojenih podtipova ćelija krvi pripremljena od krvi 10 dobrovoljaca serijski je razblažena kako bi se dobile različite koncentracije ćelijskih suspenzija. Ćelije su upotpunosti lizirane i ćelijski hemolizati su dodavani (1:20) alikvotima serumskog pula. Na ovaj način dobijeno je sedam serumskih pulova sa različitim koncentracijama interferenata. Biohemijski parametri mereni su na autoanalizatoru. Slobodni hemoglobin, kao marker hemolize, meren je spektrofotometrijski, dok su mijeloperoksidaza i β-tromboglobulin mereni enzimskim imunoodređivanjem. Procenat promene nivoa analita u serumskim pulovima procenjivan je Wilcoxon Signed Rank Testom i upoređivan sa klinički definisanim za svaki test.
Rezultati: Klinički značajne promene nađene su za laktat dehidrogenazu, kalijum, aspartat aminotransferazu, kreatin kinazu, magnezijum, ukupne proteine, ukupni holesterol, neorganski fosfat, i glukozu za eritrocite (RBC); laktat dehidrogenazu, aspartat aminotransferazu, ukupne proteine, neorganski fosfat i glukozu za trombocite (PLT). Slobodni hemoglobin je bio u značajnoj korelaciji sa RBC ($r = 0,999$; $p = 0,001$), dok mijeloperoksidaza i β-tromboglobulin nisu bili u značajnoj korelaciji sa leukocitima (WBC) i trombocitima (PLT).
Zaključak: Utvrđen je značajan efekat RBC hemolize u serumu na rutinske biohemijske testove, dok su još potrebna dodatna ispitivanja za potvrdu ovakvih efekata hemolize PLT i WBC.

Ključne reči: hemoliza, interferencija, biohemijski test, slobodan hemoglobin, mijeloperoksidaza

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List of abbreviations: β-TG, β-thromboglobulin; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca, calcium; CK, creatine kinase; fHb, free hemoglobin; HI, hemolysis index; LDH, lactate dehydronase; Mg, magnesium; MPO, myeloperoxidase; PLT, platelets; RBC, red blood cells; WBC, white blood cells.

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Introduction

Hemolyzed samples make up 3% of the routine samples sent to clinical laboratories and are responsible for 39–69% of the unsuitable specimens, this is 5 times more than the second most frequent reason (1). Although hemolysis may occur in vivo, the in vitro hemolysis in the preanalytical phase is the major problem faced by clinical laboratories (2, 3).

Elucidation of the interference mechanisms of hemolysis is necessary for more precise solutions of the problem of in vitro hemolysis. Hemolyzed samples interfere with the test results by several mechanisms such as compositional interference due to the difference between the intra- and extracellular concentration of the analytes, signal interference in instrumental measurements and chemical interference in analytical reactions. These interference mechanisms might coexist in varying combinations (4). Although there is considerable information about the occurrence mechanisms of hemolysis, no practical solution such as removal of the interferent or application of a correction factor has been established (5). The rejection of the test results of hemolyzed samples may lead to a delay in diagnosis thus threatening the patient's safety, and the request of an additional sample increases the workload and the cost (6–10).

Definitions are generally to explain sources, potent factors or mechanisms of a process and are related to viewpoints of a topic. The definition hemolysis comes from the Latin word hemo (blood) and lysis (to break open) and means destruction of blood cells (11). Scientists usually restrict hemolysis to red blood cells (RBC) called erythrolysis which makes up the greatest percent of blood cells and ignores the effects of the other blood cells. In relation to this approach, the methods to evaluate the hemolysis depends »solely« on the measured amount of free hemoglobin (fHb) released from destructed RBC (12). In contrary, a small number of scientists describe hemolysis as the destruction of all types of blood cells, namely pancytolysis and state that white blood cells (WBC) and platelets (PLT) also contribute to hemolysis (13, 14). Dimeski et al investigated the relationship between cell concentration and test results in pancytolysis and found that WBC may contribute to the increase in potassium levels in lysis state (15). The question about the separate effects of lysis of different cell types on the test results still need to be answered.

The aim of this study is to establish how the concentration and type of lysed cells contribute to hemolysis interference and to search for hemolysis markers released from blood cells other than RBC.

Materials and Methods

Study Design

This study started after the approval by the Local Research Ethical Committee (Dokuz Eylül University Medical Faculty, Izmir, Turkey) and all contributing participants in this study have given written consent. Thirteen participants aged 18–55 were included in this study. They met the blood bank donor requirements and had no pathological values in biochemistry, whole blood count or serology laboratory results. The laboratory data of participants were summarized in *Table I*.

The steps taken in this study were shown in *Figure 1*.

Preparation of Interferent

Blood Collection: One unit of whole blood (450 mL) from each participant (n=10) was collected in blood bags on different days. The blood bag system used for blood collection was Compoflex 4-bag system with Citrate Phosphate Dextrose Adenine (CPDA) solution with no filter (Fresenius Kabi AG, Bad Hamburg, Germany). Each blood collection was completed within 10 min.

Cell Separation: The whole blood samples in blood bags were processed using semi-automated blood component separator Compamat G4 (Fresenius Hemocare Inc., Redmond, Washington). RBC suspensions were separated into satellite bags with Salin-Adenin-Glucose-Mannitol (SAG-M) and PLT suspensions were separated into satellite bags without anticoagulant. Also, WBC rich residue bloods were left in primary blood bags.

Cell Isolation and Washing: Following these steps, 3 mL of WBC rich residue blood in the whole blood bag was carefully added and layered on 3 mL Histopaque 1119 in falcon tubes of 15 mL. These tubes were centrifuged at 700 × g for 30 min at unbraked stop mode and the tubes were carefully removed. The WBC rich cell suspensions were collected from the buffy coat layer using micropipette.

RBC suspensions were filtered 2 times by gravity filtration method with laboratory type WBC filters. Then, RBC suspension was added into falcon tube of 15 mL and centrifuged at 200 ×g for 10 min. 2 mL of RBC package was removed and washed with 10 mL of normal saline and this washing step was repeated for 5 times. After the final washing step, supernatant was disposed and 4 mL of normal saline was added to RBC package and the tube was overturned gently for 10 times for homogenization.

Similarly, WBC rich cell suspensions from buffy coat were centrifuged at 200 × g for 10 min. PLT rich supernatants were disposed until 2 mL volume remained. WBC rich sediments were washed with 10 mL of normal saline for 5 times. After the final washing step, the samples were combined and the tube was overturned gently for 10 times for homogenization.

Likewise, PLT suspensions were filtered by gravity filtration method with laboratory type WBC filters.

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Table I Laboratory data of 13 participants with analytical method information.

Characteristics	Method	Mean ± SD
WBC × 10^9 (cell/L)	Electrical impedance method	6.05 ± 1.51
RBC × 10^{12} (cell/L)	Electrical impedance method	5.07 ± 0.36
PLT × 10^9 (cell/L)	Electrical impedance method	208 ± 40
Hb (g/L)	Spectrophometric method with noncyanide reagent	151 ± 9
Glucose (mmol/L)	Enzymatic method with he×okinase	5.22 ± 0.99
Blood Urea Nitrogen (mmol/L)	Enzymatic method with urease/glutamate dehydrogenase	4.64 ± 1.07
Creatinine (μmol/L)	Jaffe kinetic method	70.7 ± 8.8
Uric Acid (μmol/L)	Enzymatic method with uricase/pero×idase	321.2 ± 71.4
AST (U/L)	IFCC method without pyrido×al phosphate activation	18 ± 2
ALT (U/L)	IFCC method without pyrido×al phosphate activation	17 ± 4
ALP (U/L)	IFCC method with 2-amino-2-methyl-1-propanol (AMP) buffer	73 ± 17
CK (U/L)	IFCC method with N-acetylcysteine-activated	90 ± 31
GGT (U/L)	IFCC method with L- -glutamyl-3-carbo×y-4-nitroanilide	19 ± 8
LDH (U/L)	IFCC method with lactate-pyruvate	142 ± 17
Total Cholesterol (mmol/L)	Enzymatic method with cholesterol esterase	4.76 ± 0.72
Triglyceride (mmol/L)	Enzymatic metod with glycerol phosphate o×idase	1.50 ± 0.72
HDL (mmol/L)	Direct method with immunoinhibition	1.90 ± 0.23
LDL (mmol/L)	Direct method with selective detergent	2.51 ± 0.67
Total Protein (g/L)	Biuret method	69 ± 3
Albumin (g/L)	Bromocresol green method	46 ± 2
Total Bilirubin (μmol/L)	Diazo method, 3,5-dichlorophenyldiazonium tetrafluoroborate (DPD)	13.85 ± 4.28
Direct Bilirubin (μmol/L)	Diazo method, 3,5-dichlorophenyldiazonium tetrafluoroborate (DPD)	2.39 ± 1.03
Calcium (mmol/L)	Arsenazo III method	2.42 ± 0.10
Inorganic Phosphate (mmol/L)	Ammonium molybdate UV method	1.07 ± 0.13
Iron (μmol/L)	Tri-pyridyl-triazine method	18.4 ± 7.2
UIBC (μmol/L)	Nitroso-PSAP method	53.3 ± 11.5
Magnesium (mmol/L)	×ylidyl blue method	0.82 ± 0.05
Sodium (mmol/L)	Indirect ion-selective electrode method	140 ± 1
Potassium (mmol/L)	Indirect ion-selective electrode method	3.99 ± 0.29
Chloride (mmol/L)	Indirect ion-selective electrode method	104 ± 2

Serological tests applied: HBs Ag, Anti-HCV, HIV Ag/Ab, Anti Treponema pallidum IgM, IgG-, were negative. AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; ALP, Alkaline Phosphatase; CK, Creatine Kinase; fHb, free hemoglobin; GGT, Gamma-glutamyl transferase; HDL, High Density Lipoprotein; LDH, Lactate Dehydrogenase; LDL, Low Density Lipoprotein; PLT, platelet; RBC, red blood cell; UIBC, Unsaturated iron-binding capacity, WBC, white blood cell.

Then, PLT suspensions were added into 15 mL of falcon tubes and centrifuged at 200 × g for 10 min. 3 mL of PLT rich supernatants were transferred into different falcon tubes and the tube was overturned gently for 10 times for homogenization.

Yet, no washing was applied to PLT suspensions because of aggregation problem.

Cell Suspension Preparation: The concentrations of the cell suspensions were measured by LH780 hematology analyzer (Beckman Coulter Inc., Miami, Florida, USA). The linearity ranges of this instrument for RBC, WBC, PLT were 0–8 × 10^6; 0–400 × 10^3; 0–3000 × 10^3/μL, respectively. The cell suspensions were diluted using normal saline to obtain the con-

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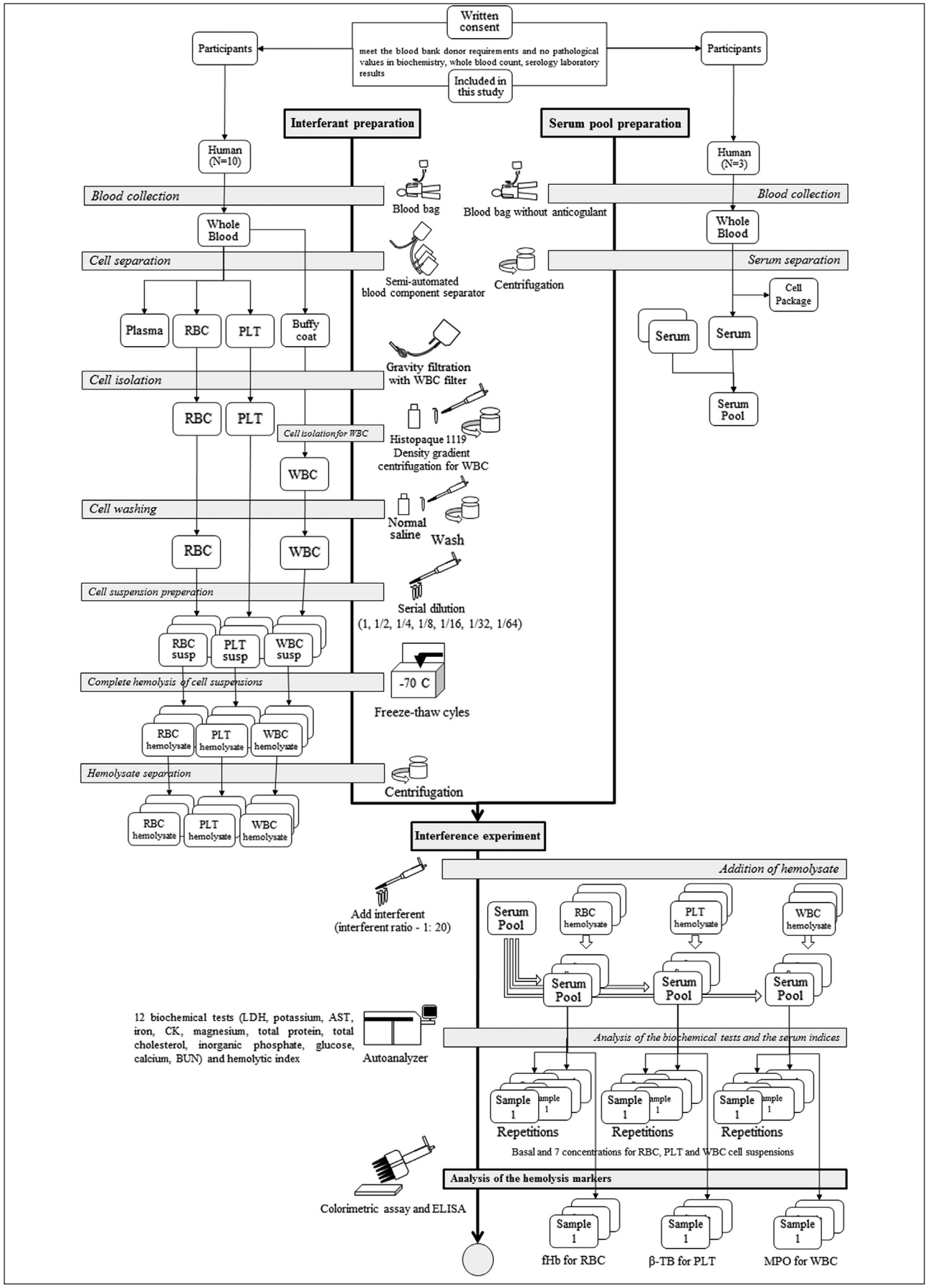


Figure 1 The steps of the study.

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centrations of 1600000/μL for RBC, 10000/μL for WBC and 96000/μL for PLT. Then, cell suspensions were diluted serially (1/2, 1/4, 1/8, 1/16, 1/32, 1/64) in micro-centrifuge tubes. The samples in micro-centrifuge tubes were stored at –70 °C.

Complete Hemolysis of Cell Suspension: The separated cell suspensions were kept at –70 °C for 1 week and then blood cells were lysed completely by freeze-thaw cycles (16). The overall hemolysis of the cell suspensions were confirmed by microscopic examination of hemolysates containing the lowest and the highest cell concentrations for 3 cell types, using Neubauer cell counting chamber and smear prepared by Wright stain.

Hemolysate Separation: The cell debris was separated by centrifugation at 12000 × g for 10 min. Supernatants were removed and added into micro-centrifuge tubes as hemolysate for the interference experiment.

Serum Pool Preparation

Blood Collection: One unit of whole blood (450 mL) was drawn into blood bags without anticoagulant from 3 participants. Blood bags without anticoagulant had been obtained using sterile anastomosis. Each blood collection was completed within 10 min.

Serum Separation: Blood samples were allowed to clot for 90 min at a 45° angle and centrifuged at 1200 × g for 10 min at unbraked stop mode. Serum samples were transferred by Pasteur pipette into an Erlenmeyer flask. These procedures were done on the last 2 days before the interference experiment and the samples were stored at +4 °C.

Interference Experiment

Addition of Hemolysate: To obtain 1/20 interferent ratio, 950 μL serum pool and 50 μL hemolysate which consisted of lysed cells at different concentrations were added to each micro-centrifuge tube and then vortexed. For the basal concentration, instead of hemolysate, 50 μL normal saline was added and then vortexed.

Analysis of The Biochemical Tests and The Serum Indices: The 12 biochemical test parameters were selected according to the degree of susceptibility to hemolysis as classified by the manufacturer: 4 from 50-99 of hemolysis index (HI) (LDH, potassium, AST, iron), 2 from 100–199 of HI (CK, Mg), 2 from 200–299 of HI (total protein, total cholesterol), 2 from 300–500 of HI (inorganic phosphate, glucose) and 2 from >500 of HI (Ca, BUN) (17). The biochemical tests were measured in duplicate by using the samples which were divided into two aliquots within a single analytical run.

All the samples were analyzed with AU5800 Clinical Chemistry System (Beckman Coulter Inc., Miami, Florida, USA) using original reagents. The assay methods used were summarized in *Table I*. All biochemical tests were analyzed at the Central Laboratory of Dokuz Eylül University Hospital, an ISO 15189 accredited laboratory. All results were reported as the mean of duplicated measurements.

The effect of hemolysis on the biochemical tests was evaluated by comparing 7 different concentrations of each blood cell type with the basal concentration (*Table II*). The semi-quantitative equivalents of fHb were calculated for a well matched comparison of the clinical thresholds determined by this study against other studies as well as with the data reported by the manufacturer of the analytical system used (*Table III*).

Table II The cell components of the separated RBC, PLT and WBC suspensions.

Concentrations	RBC suspensions		PLT suspensions		WBC suspensions	
	RBC* conc.	Cell distribution** RBC:WBC:PLT	PLT*conc.	Cell distribution** RBC:WBC:PLT	WBC* conc.	Cell distribution** RBC:WBC:PLT
Basal	0	0 : 0 : 0	0	0 : 0 : 0	0	0 : 0 : 0
1	20000	95.2 : 0 : 4.8	2000	0 : 2.4 : 97.6	100	0 : 9.1 : 90.9
2	50000	98.0 : 0 : 2.0	4000	0 : 0 : 100	200	0 : 16.7 : 83.3
3	100000	99.0 : 0 : 1.0	6000	0 : 0 : 100	350	0 : 14.9 : 85.1
4	200000	99.5 : 0 : 0.5	12000	0 : 0.4 : 99.6	800	0 : 21.1 : 78.9
5	400000	99.8 : 0 : 0.2	23000	0 : 0 : 100	1900	59.2 : 11.2 : 29.6
6	800000	99.9 : 0 : 0.1	44500	0 : 0.2 : 99.8	4700	44.1 : 20.7 : 35.2
7	1620000	99.9 : 0 : 0.1	90000	0 : 0.1 : 99.9	10500	44.0 : 23.1 : 33.0

Conc, concentration; PLT, platelet; RBC, red blood cell; WBC, white blood cell.
*Cell concentrations are shown as median (cell/μL).
**Cell distribution rates are shown as percent (%).

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Table III Median fHb concentrations of RBC suspensions and corresponding hemolysis index ranges of AU5800 Clinical Chemistry System.

Manufacturer'shemolysis indexranges		fHb concentrations of RBC suspensions*							
		Basal	RBC1	RBC2	RBC3	RBC4	RBC5	RBC6	RBC7
N	<0.50	0.082	0.132	0.234	0.346	–	–	–	–
1+	0.50–0.99	–	–	–	–	0.526	–	–	–
2+	1.00–1.99	–	–	–	–	–	1.210	–	–
3+	2.00–2.99	–	–	–	–	–		2.263	–
4+	3.00–5.00	–	–	–	–	–	–	–	4.703
5+	>5.00	–	–	–	–	–	–	–	–

fHb, free hemoglobin; N, normaL; RBCs, red blood cells. * fHb levels are shown as g/L.

Table IV The percent changes and percent bias limits of 12 biochemical tests for each interferent concentration.

Test		Concentrations								Bias limit (%)
	Basal	1	2	3	4	5	6	7		
Cell concentration (cell/µL)	RBC PLT WBC	–	20000 2000 100	50000 4000 200	100000 6000 350	200000 12000 800	400000 23000 1900	800000 44500 4700	1620000 90000 10500	–
Hemolysis Markers		Concentrations**								
fHb (g/L) β-TB (ng/mL) MPO (ng/mL)	RBC PLT WBC	0.082 9.1 41.3	0.132 10.3 39.6	0.234 10.3 39.6	0.346 12.3 42.4	0.526 12.2 40.0	1.210 11.4 40.6	2.263 10.6 40.0	4.703 8.7 40.1	–
Biochemical Tests		Conc.	Percent Changes (%)							
Lactate Dehydrogenase (U/L)	RBC PLT	151	+3.2* +0.3	+4.8* –0.2	+10.0* +0.3	+21.9* +1.3	+43.9* +1.6	+91.5* +3.8*	+190.5* +7.9*	±4.3
Potassium (mmol/L)	RBC PLT	3.49	+0.1 –0.1	+0.6 –0.1	+1.3 –0.2	+1.9* –0.1	+4.9* +0.1	+9.7* +0.1	+19.7* +1.0	±1.8
Aspartate Aminotransferase (U/L)	RBC PLT	18	0.0 +2.9	+0.1 +1.4	+8.6 0.0	+8.6* +2.7	+22.5 *0.0	+35.1* +2.9	+74.3* +5.7*	±6.5**
Iron (µmol/L)	RBC PLT	16.7	0.0 –0.5	+0.5 +0.5	0.0 +0.3	–0.5 +0.5	0.0 –0.3	0.0 +0.3	–1.1 +1.1	±8.8
Creatine Kinase (U/L)	RBC PLT	104	0.0 +0.5	–0.2 +0.5	0.0 +0.7	0.0 +1.4	+2.4 +0.7	+6.4 *0.7	+13.5* +1.7	±11.5
Magnesium (mmol/L)	RBC PLT	0.80	–0.3 0.0	0.0 +0.6	+0.6 0.0	+0.9 0.0	+1.9* +0.6	+4.4* +0.3	+9.4* +0.6	±1.8
Total protein (g/L)	RBC PLT	71	+0.1 0.0	0.0 –0.1	+0.3 +0.2	+0.6 –0.2	+0.9 0.0	+1.9* +0.5	+3.3* +1.5*	±1.4**
Total cholesterol (mmol/L)	RBC PLT	4.14	+0.3 0.0	+0.5 +0.2	+0.5 0.0	+1.6 +0.5	+2.2* +0.3	+4.0* +0.5	+7.9* +1.1	±4.1
Inorganic phosphate (mmol/L)	RBC PLT	0.87	0.0 0.0	0.0 0.0	0.0 0.0	+1.9 0.0	+3.7* 0.0	+7.4* +1.9	+18.5* +3.7*	±3.4**
Glucose (mmol/L)	RBC PLT	4.33	0.0 +0.6	+0.3 +1.3	0.0 +1.0	+0.6 +1.9	–0.6 +1.9*	–0.6 +2.9*	–0.6 +4.9*	±2.4**
Calcium (mmol/L)	RBC PLT	2.33	0.0 +0.1	0.0 +0.2	–0.2 0.0	+0.2 +0.1	+0.1 –0.2	0.0 +0.1	0.0 +0.4	±0.8
Blood Urea Nitrogen (mmol/L)	RBC PLT	3.79	–0.2 +0.5	+0.2 –0.5	+0.2 –0.2	+1.2 +0.2	+1.2 +1.2	+1.9* 0.0	+2.8* +1.6	±5.6**

β-TG, thromboglobulin; fHb, free hemoglobin; MPO, myeloperoxidase. The biochemical tests are listed according to the degree of sensibility to hemolysis as indicated by the manufacturer. Results exceeding the bias limit are marked in bold. Cell concentrations, hemolysis markers and biochemical tests are presented as median.*: $p < 0.007$ (significance according to basal concentrations). ** Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. Desirable specification for total error, imprecision, and bias, derived from intra- and inter- individual biologic variation. The 2014 update. Available from: www.westgard.com/biodatabase1.htm. Accessed October, 2016.

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Table V Comparison of clinical thresholds to previous studies and the manufacturer data based on whole blood cell lysis.

Biochemical Tests	Whole blood lysis						Separated blood lysis				
	Lippi et al. Clinical thresholds		Köseo lu et al. Clinical thresholds		Manufacturer data Cut off limits		RBC lysis Clinical thresholds			PLT lysis Clinical thresholds	
	fHb		fHb		fHb		fHb		RBC Conc	β-TG	PLT Conc
Lactate Dehydrogenase	0.160	1+	0.270	1+	0.50–0.99	2+	0.234	1+	50000	8.7	90000
Potassium	0.600	2+	0.270	1+	0.50–0.99	2+	0.526	2+	200000	–	–
Aspartate Aminotransferase	0.300	1+	0.270	1+	0.50–0.99	2+	0.346	1+	100000	8.7	90000
Iron	5.100	6+	–	Ø	0.50–0.99	2+	–	Ø	–	–	–
Creatine Kinase	1.300	3+	1.270	3+	1.00–1.99	3+	4.703	5+	1620000	–	–
Magnesium	2.600	4+	3.340	5+	1.00–1.99	3+	1.210	3+	400000	–	–
Total protein	ND**		3.340	5+	2.00–2.99	4+	2.263	4+	800000		8.7
Total cholesterol	ND**		3.340	5+	2.00–2.99	4+	4.703	5+	1620000	–	–
Inorganic phosphate	5.100	6+	1.270	3+	3.00–5.00	5+	1.210	3+	400000	8.7	90000
Glucose	–	Ø	0.750	2+	3.00–5.00	5+	–	Ø	–	10.6	44500
Calcium	–	Ø	–	Ø	>5.00	6+	–	Ø	–	–	–
Blood Urea Nitrogen	20.600	6+	–	Ø	>5.00	6+	–	Ø	–	–	–

β-TG, thromboglobulin; Conc, concentration; fHb, free hemoglobin; PLT, platelet; RBC, red blood cell. Parameters which show significantly different clinical thresholds in whole blood lysis compared to RBC lysis are marked in bold. *: Cell concentrations, fHb, β-TB and MPO were presented as cell/μL, g/L, ng/mL and ng/mL, respectively. Semi quantitative evaluation: limit is not exceeded »Ø«. fHb <0.50 g/L »1+«, fHb =0.50–0.99 g/L »2+«, fHb =1.00–1.99 g/L »3+«, fHb =2.00–2.99 g/L »4+«, fHb =3.00–5.00 g/L »5+«, fHb >5.00 g/L »6+«. **: ND: Not detected

The percent changes from basal concentrations were calculated for each biochemical tests via this formula: Percent change in test result (%) = [(Experiment sample test result – Basal sample test result) / Basal sample test result] × 100. The percent change values of biochemical tests were compared with the percent bias limit. The analytical threshold, shown in *Table IV*, is the concentration at which the statistically significant difference begins and the clinical threshold is the one at which the percent bias limit is exceeded. The percent bias limits are from a large scaled database research that considers the between and within subjects biological variation and is made by »Analytical Quality Commission from the Spanish Society of Clinical Chemistry and Molecular Pathology (SEQC)« (*Table IV–V*) (18). The data of some percent bias limits is used as updated from Westgard Website (19).

Analysis of the Hemolysis Markers

fHb, as hemolysis marker for RBC, was measured with a colorimetric assay kit (Catachem Inc., Oxford, England). fHb determination method was based upon the peroxidase activity of hemoglobin. Hemoglobin activates the oxidation of 3.3′, 5.5′-tetramethylbenzidine by hydrogen peroxide to form a chromogenic product with maximum absorption at 650 nm (20).

Myeloperoxidase (MPO), as hemolysis marker for WBC, was measured with an enzyme immunoassay test kit (Biocheck Inc., Foster City, California, USA). And finally, β-thromboglobulin (β-TG), as hemolysis marker for PLT, was measured with an enzyme immunoassay test kit (CUSABIO Inc., Wuhan, China) (21, 22).

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Statistical Analysis

Statistical analyses were performed by using Statistical Package for Social Sciences (SPSS) software version 22.0 for Windows (version 22, SPSS Inc., Chicago, IL, USA) and Microsoft Office Excel 2013 (Microsoft, Seattle, WA, USA). The distribution of the variables were determined using Shapiro-Wilk Test and P value of >0.05 was considered as normal (Gaussian) distribution. Descriptive statistics were presented with median for all variables. Friedman Variance Analysis was used for comparing more than two groups. Statistical significance of the change from baseline was measured using the Wilcoxon Signed Rank Test (P-value was adjusted to 0.007 for pairwise comparisons). Simple Linear Regression was used for association between blood cell concentrations and hemolysis markers and and P values of <0.05 were considered significant.

Results

Evaluation of the Interferent Preparation

Evaluation of the Cell Isolation: The composition of the cell suspensions that are prepared after the blood component separation, detected by whole blood cell count, is shown in *Table II*. Considering the limitations of the blood component separation methods used, purity of 95% was aimed and obtained for RBC and PLT suspensions. But this goal could not be achieved for the WBC suspensions. The data about WBC is not presented in this study, because of WBC suspensions were contaminated with RBC and PLT to a large extent.

Evaluation of the Cell Lysis: In the microscopic examination of the hemolysates of the lowest and the highest cell concentrations for 3 cell types no intact cell was detected, confirming that complete hemolysis was achieved. The degree of hemolysis for RBC suspensions after the hemolysis procedure were evaluated according to fHb amounts and matched with the ranges of the hemolysis indices of the AU5800 Clinical Chemistry System (*Table III*).

Evaluation of the Interference

The effect of hemolyzed RBC and PLT suspensions to the biochemical tests is shown as percent changes in *Table IV*. The percent change values were compared with the clinical threshold. The percent change values exceeding the clinical threshold were determined as +4.8% for LDH at 0.234 g/L of fHb level (or 50000 cell/μL of RBC), +1.9% for K at 0.526 g/L of fHb level (or 200000 cell/μL of RBC), +8.6% for AST at 0.346 g/L of fHb level (or 100000 cell/μL of RBC), +1.9% for Mg and +3.7% for i.phosphate at 1.210 g/L of fHb level (or 400000 cell/μL of RBC) and +7.9% for LDH, +1.5% for total protein, +3.7% for inorganic phosphate, and +2.9% for glucose at 90000 cell/μL of PLT.

Evaluation of the Hemolysis Markers

There was a positive linear association between RBC concentration and fHb (fHb = 5.12 + 0.28 × RBC count). 99% of variations between RBC concentration and fHb are explained by this equation ($P=0.001$). There was no association between WBC concentration and MPO and between PLT concentration and β-TG ($P=0.436$; $P=0.277$ respectively).

Discussion

It has been reported many times in literature that hemolysis interference causes bias in a great variety of tests including biochemical, immunochemical, hematological, coagulation and blood gas analysis. This problem occurs very frequently in clinical laboratories and threatens the patients' safety.

In this study, the effect of hemolysis on biochemical tests have been investigated by an experimental model focusing on blood cell subtypes. The clinical thresholds for biochemical tests were evaluated semi-quantitatively and compared to the data reported by previous studies in literature and also to the manufacturer of the analytical system used (*Table V*). There are two studies, Köseoğlu (23) and Lippi (24), which examine the relationship between fHb and the affected test results using similar biochemical tests and bias limits. The clinical thresholds showed similarities between two studies and manufacturer data for only three parameters (LDH, AST and calcium). On the other hand, CK showed significantly higher values whereas magnesium showed significantly lower clinical thresholds compared to both previous studies. Manufacturers' suggestions about the use of specified cut off limits for the test results of hemolyzed specimens must also be regarded (17). The cut off limits were identical or similar to LDH, potassium, AST, magnesium, total protein and total cholesterol while the cut off limits were markedly different for iron, CK, inorganic phosphate and glucose. It should be noticed that these clinical studies' and manufacturer's data reflect whole blood lysis results whereas our study shows separated RBC lysis results (17, 23, 24).

Other factors that further decrease the comparability of these interference studies are variations regarding sample type, the method of hemolysis, dilution factor, analytical methods, evaluation limits and statistical methods (17, 23–27). Further supporting this opinion, Grafmeyer et al. (25) investigated the effects of hemolysis, icterus and lipemia on 20 different biochemical tests using 15 automatic analysers representative of those found on the French market. He stated that the difference in methods as well as the difference in adaptation of the methods to the

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analyzers are important. Considering the importance of the analytical method in interference, it is irrational to accept the results of studies performed with a specific analytical system and adapt it to all clinical laboratories. Thus, in routine clinical chemistry testing it might be appropriate for each laboratory to determine its own clinical thresholds (23, 24).

In order to achieve standardization in the studies on hemolysis interference, some critical suggestions have been put forward by Lippi (24). According to Lippi, the method used for preparation of hemolysate must reflect the mechanical hemolysis during phlebotomy, the interferent concentrations must be in the range of hemolysis observed in clinical specimens, the interferent must be added in the smallest dilution possible, the bias related to interference must be compared with the clinical threshold and the analyte-interferent relationship data must be evaluated (28). We did not preferred osmotic shock and similar mechanical destruction methods in this study considering the disadvantages related to the addition of chemicals resulting in higher dilution and contamination. Also, we used the freeze-thaw method to achieve full lysis of the isolated cells since the main objective was to evaluate the relationship between the concentration of the lysed cells and the analyte. Supported by Lippi's article and evaluating that Meites has stressed that the serum dilution ratio should be kept at minimum (not more than 5%) in interference experiments, the sample matrix of this study was preserved by using the ratio of 1/20 for the hemolysate (28, 29).

As to the effect of the PLT on the biochemical tests; the clinical threshold for the affected tests (LDH, AST, total protein, inorganic phosphate and glucose) was exceeded only at high concentrations of hemolysis (*Table IV–V*). This may indicate that the contribution of PLT is rather low in whole blood lysis. On the other hand, if hemolysis occurs in a patient with high PLT count, the effect of PLT on these tests may emerge more apparently. More intriguing is the effect of the PLT on potassium levels, although full lysis of PLT has been achieved in our experimental model the clinical threshold for potassium was not exceeded (*Table IV–V*). In the study of Dimeski et al. (15), the change in the level of potassium have been investigated per unit hemolysis index (HI) at varying blood cell (RBC, WBC, PLT) concentrations and it was also concluded that platelet count did not significantly contribute to potassium increase.

One of the limitations of this study was the possible artificial activation of PLT which leads to aggregation and the release of cell content. The aggregation of cells hampers the cells, thus the measurement of cell concentration and preparation of cell suspensions with accurate cell count are not possible. On the other hand, the release of cell content affects test results in interference experiments, here mainly the release of potassium from PLT's gave unexpected results. From this point of view, there exists a controversy between the application of aggressive procedures for satisfactory purity and gentle procedures for reliable interference experiments. In this study, no washing step was applied for PLT to avoid the centrifugation which may lead to aggregation and no chemicals such as antiaggregant were used to avoid contamination and a relatively gentle procedure was applied in this experiment.

Another limitation of this study occurred with WBC's separation. The quadruple blood bag system and the semi-automated blood component separator verified by the requirements of the blood bank was used for cell separation. This study specifically applied additional isolation steps such as filtration for RBC and PLT and Histopaque 1119 density centrifugation for WBC. In low concentrations, the purity that could be achieved was 95% for RBC, 97% for PLT and <95% for WBC. Since WBC could not be sufficiently isolated, its effect on biochemical tests could not be exactly evaluated. In the future, more sufficient purification will allow isolation of mature cells. Such cells as mononuclear cells like lymphocyte, monocyte, or polymorphonuclear cells like neutrophil, basophil and eosinophil for WBC can be isolated. Isolation of immature cells such as reticulocyte, nucleated cells and erythroblast for RBC and giant PLT for PLT are also possible. Even more, pathological cells such as those seen with leukemia and lymphoma cells for WBC can be isolated. Thus, this can make it possible to exhibit the effects of WBC on hemolysis and also the effect of hemolysis even in hematological disorders. Simulation of the hemolysis of the specimens representing individuals with increased numbers of blood cells in cases of polycythemia, thrombocytosis and leukocytosis, need to be performed in further interference experiments.

The main focus of this study was on how the number of lysed cells affect the change in the test results. Unfortunately, this approach is not applicable in routine practice. It is more convenient to measure biomarkers released following cell lysis to indicate the degree of hemolysis. In the studies about hemolysis interference, test results are commonly evaluated against fHb as an indicator of hemolysis. Many clinical laboratories perform automatic spectrophotometric measurement of fHb instead of the subjective visual inspection in order to determine the degree of hemolysis (30). fHb levels show very strong positive correlation with the number of lysed RBC, as it is also observed in this study. However, it should be realized that fHb levels do not reflect the contribution of other blood cells to hemolysis. In the endeavor to discover potential biomarkers of hemolysis, MPO and β-TG were investigated respectively for WBC and PLT. No significant correlation could be detected between these molecules and the number of lysed cells possibly due to the matrix effect related to the addition of

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hemolysate, though in minimum amounts, to the serum. If specific reliable biomarkers for different blood cells could be discovered, the grading of hemolysis based only on fHb can be modified.

In this study, the relationship between RBC concentration and the effects on biochemical tests are clearly established. Yet, it cannot be ignored that additional studies are required in order to verify this relationship for PLT and WBC concentrations. The methods for isolation of WBC and PLT need to be improved in order to establish their influence on biochemical tests in hemolyzed samples more definitely. These studies will provide valuable clues for solutions of the hemolysis interference in clinical laboratories. It may be foreseen that search for biomarkers of hemolysis for different blood cells will be a significant objective for future studies.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References

1. Lippi G, Guidi GC. Risk management in the preanalytical phase of laboratory testing. Clin Chem Lab Med 2007; 45: 720–7.
2. Giménez-Marín A, Rivas-Ruiz F, del Mar Pérez-Hidalgo MDM, Molina-Mendoza P. Pre-analytical errors management in the clinical laboratory: A five-year study. Biochem Med (Zagreb) 2014; 24: 248–57.
3. Lipi G, Cadamuro J. Novel opportunities for improving the quality of preanalytical phase. A glimpse to the future?J Med Biochem 2017; 36: 293–300.
4. Kroll MH, Elin RJ. Interference with clinical laboratory analyses. Clin Chem 1994; 40: 1996–2005.
5. Lippi G, Avanzini P, Pavesi F, Bardi M, Ippolito L, Aloe R, et al. Studies on in vitro hemolysis and utility of corrective formulas for reporting results on hemolyzed specimens. Biochem Med (Zagreb) 2011; 21: 297–305.
6. Cadamuro J, Wiedemann H, Mrazek C, Felder TK, Oberkofler H, Fiedler GM, et al. The economic burden of hemolysis. Clin Chem Lab Med 2015; 53: e285–8.
7. Gunnur Dikmen Z, Pinar A, Akbiyik F. Specimen rejection in laboratory medicine: Necessary for patient safety? Biochem Med (Zagreb) 2015; 25: 377–85.
8. Jacobs P, Costello J, Beckles M. Cost of haemolysis. Ann Clin Biochem 2012; 49: 412.
9. Lippi G, Cervellin G, Favaloro EJ, Plebani M. In Vitro and In Vivo Hemolysis: An Unresolved Dispute in Laboratory Medicine, Walter de Gruyter GmbH & Co. KG, Berlin/Boston 2012.
10. Howanitz PJ, Lehman CM, Jones BA, Meier FA, Horowitz GL. Clinical Laboratory Quality Practices When Hemolysis Occurs. Arch Pathol Lab Med 2015; 139: 901–6.
11. Hawkins R. »Hemolysis« – toward clearer terminology. Arch Pathol Lab Med 2010; 134: 1104.
12. Simundic A-M, Topic E, Nikolac N, Lippi G. Hemolysis detection and management of hemolyzed specimens. Biochem Med (Zagreb) 2010; 20: 154–9.
13. Baer DM, Ernst DJ, Willeford SI, Gambino R. Investigating elevated potassium values. Medical Laboratory Observer 2006; 38: 24, 6, 30–1.
14. Guder WG, Narayanan S, Wisser H, Zawta B. Samples: From the Patient to the Laboratory: The impact of preanalytical variables on the quality of laboratory results, third ed. ed., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 2003.
15. Dimeski G, Clague AE, Hickman PE. Correction and reporting of potassium results in haemolysed samples. Ann Clin Biochem 2005; 42: 119–23.
16. Lippi G, Musa R, Aloe R, Mercadanti M, Pipitone S. Influence of temperature and period of freezing on the generation of hemolysate and blood cell lysate. Clin Biochem 2011; 44: 1267–9.
17. Theodorsson E. Quality assurance in clinical chemistry: a touch of statistics and a lot of common sense. J Med Biochem 2016; 35: 103–12.
18. Ricós C, Alvarez V, Cava F, García-Lario JV, Hernández A, Jiménez CV, et al. Current databases on biological variation: pros, cons and progress. Scand J Clin Lab Invest 1999; 59: 491–500.
19. Westgard QC. Desirable Biological Variation Database specifications. Available from:https://www.westgard.com/biodatabase1.htm (Accessed: 09- Aug- 2017).
20. Levinson SS, Goldman J. Measuring hemoglobin in plasma by reaction with tetramethylbenzidine. Clin Chem 1982; 28: 471–4.
21. Gurney D, Lip GYH, Blann AD. A reliable plasma marker of platelet activation: Does it exist? American Journal of Hematology 2002; 70: 139–44.
22. Pulli B, Ali M, Forghani R, Schob S, Hsieh KLC, Wojtkiewicz G, et al. Measuring Myeloperoxidase Activity in Biological Samples. PLoS One 2013; 8: e67976.
23. Koseoglu M, Hur A, Atay A, Cuhadar S. Effects of hemolysis interferences on routine biochemistry parameters. Biochem Med (Zagreb) 2011; 21: 79–85.
24. Lippi G, Salvagno GL, Montagnana M, Brocco G, Guidi GC. Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med 2006; 44: 311–6.
25. Grafmeyer D, Bondon M, Manchon M, Levillain P. The influence of bilirubin, haemolysis and turbidity on 20 analytical tests performed on automatic analysers. Results of an interlaboratory study. Eur J Clin Chem Clin Biochem 1995; 33: 31–52.
26. Jeffery J, Sharma A, Ayling RM. Detection of haemolysis and reporting of potassium results in samples from neonates. Ann Clin Biochem 2009; 46: 222–5.

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27. Owens H, Siparsky G, Bajaj L, Hampers LC. Correction of factitious hyperkalemia in hemolyzed specimens. The American journal of emergency medicine 2005; 23: 872–5.

28. Lippi G. Interference studies: focus on blood cell lysates preparation and testing. Clin Lab 2012; 58: 351–5.

29. Meites S. Letter: Reproducibly simulating hemolysis, for evaluating its interference with chemical methods. Clin Chem 1973; 19: 13–9.

30. Lippi G, Luca Salvagno G, Blanckaert N, Giavarina D, Green S, Kitchen S, et al. Multicenter evaluation of the hemolysis index in automated clinical chemistry systems. Clin Chem Lab Med 2009; 47: 934–9.

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