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Scoring System for Detecting Spurious Hemolysis in Anticoagulated Blood Specimens

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Background: The identification of *in vitro* hemolysis (IVH) using a hematology analyzer is challenging because centrifugation of the specimens cannot be performed for cell counts. In the present study, we aimed to develop a scoring system to help identify the presence of hemolysis in anticoagulated blood specimens.

Methods: Thirty-seven potassium EDTA anticoagulated blood specimens were obtained, and each specimen was divided into 3 aliquots (A, B, and C). Aliquots B and C were mechanically hemolyzed by aspirating 2 and 5 times, respectively, using a 27-gauge needle and then tested; aliquot A was analyzed immediately without any hemolysis. After the cells were counted, aliquots B and C were centrifuged and the supernatants were tested for the hemolytic index and lactate dehydrogenase levels.

Results: The 4 hematologic parameters were selected and scored from 0 to 3 as follows: <34.0, 34.0-36.2, 36.3-38.4, and ≥38.5 for mean cell hemoglobin concentration (MCHC, g/dL); <0.02, 0.02, 0.03, and ≥0.04 for red blood cell ghosts (10^{12}/L); <0.13, 0.13-0.38, 0.39-1.30, and ≥1.31 for difference value (g/dL) of measured hemoglobin and calculated hemoglobin; and <0.26, 0.26-0.95, 0.96-3.34, and ≥3.35 for difference value (g/dL) of MCHC and cell hemoglobin concentration mean. The hemolysis score was calculated by adding all the scores from the 4 parameters. At the cutoff hemolysis score of 3, the IVH of aliquots B and C were detected as 64.9% and 91.9%, respectively.

Conclusions: The scoring system might provide effective screening for detecting spurious IVH.

Key Words: Hemolysis, Score, System, Anticoagulants, Analyzer

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INTRODUCTION

The presence of hemolysis, icterus, and lipemia (HIL) in clinical specimens is known to interfere with the accurate measurement of various analytes. The identification of clinical specimens with HIL is desirable when laboratories have the resources for reducing or eliminating some of these preanalytical interferents. The main method for detecting and reporting HIL interference has involved the inspection of individual specimens by laboratory personnel. At present, the development of sophisticated chemistry analyzers has enabled the automatic detection of HIL status and reporting of HIL index values [1, 2].

However, the identification of HIL in specimens for hematology analysis is challenging because centrifugation of the specimens cannot be performed for cell counts. It is important to detect *in vitro* hemolysis (IVH) because it is the most frequently encountered during HIL interference [3]. The presence of IVH in a specimen being examined with a hematology analyzer may result in a spurious increase in measured Hb (mHb) concentration and platelet (PLT) count and a spurious decrease in red blood cell (RBC) count, Hct, and mean cell volume (MCV) [4]. An abnormality in one of these measured parameters eventually leads to abnormal calculated RBC indices, such as mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) [4]. Therefore, laboratory applications of sensitive and reliable hematologic flags or indices for identifying clinically significant

IVH might reduce laboratory turnaround time and recheck and/or recollection of specimens.

In the present study, we aimed to investigate the hemolytic effects on various hematologic parameters to determine the hematologic parameters that are indicative of clinically significant IVH. Moreover, we developed a scoring system that is sensitive and effective for detecting IVH.

METHODS

1. Laboratory analyses

This prospective study was performed during the period of December 2012 by using 37 consecutive fresh human blood specimens that were anticoagulated with EDTA (BD Vacutainer K_2 EDTA 5.4 mg, REF 367835, Becton Dickinson, Franklin Lakes, NJ, USA). All EDTA blood specimens were obtained during medical check-ups. Each specimen was divided into 3 aliquots. The first aliquot (A) was immediately tested, and the second and third aliquots (B and C, respectively) were tested after being mechanically hemolyzed by aspirating two and five times, respectively, using a 27-gauge needle. For all specimens, complete blood cell count (CBC) and automated differential count were obtained by the Advia 2120i (Siemens Healthcare Diagnostics, Sacramento, CA, USA) CBC/Diff mode. After the cells were counted, aliquots B and C were centrifuged and the supernatants were tested for hemolytic index (HI) and lactate dehydrogenase (LDH) levels using Modular DPE system (Roche Diagnostics, Bazel, Switzerland).

2. Statistical analyses

Data were analyzed by using IBM SPSS Statistics version 20 (SPSS Inc., Chicago, IL, USA). The [B-A] data set was obtained by subtracting the results of aliquot A from those of aliquot B, and the [C-A] data set was obtained by subtracting the results of aliquot A from those of aliquot C. Linear regression analysis was performed for the [B-A] and [C-A] data sets to define the correlation coefficients between objective hemolysis parameters (HI, LDH) and hematologic parameters, respectively. A paired t-test was performed to compare the mean difference of hematologic parameters between non-hemolyzed and hemolyzed specimens. A Kolmogorov-Smirnov test was performed to verify the assumption of normality.

Procedures for selecting sensitive and reliable variables are described below. First, the higher correlation coefficients ($P<0.05$) between hematologic and objective hemolysis parameters (HI, LDH) were selected from both [B-A] and [C-A] data sets. Second, we selected hematologic parameters in which the data ranges between the hemolyzed and non-hemolyzed specimens did not overlap. Third, hematologic parameters with normality ($P>0.05$) and mean values that were statistically different between the [B-A] and [C-A] data sets ($P<0.05$) were selected.

RESULTS

The mean±SD values of the HI for aliquots B and C were 1,121±147 and 3,298±328, respectively. The mean±SD values of the LDH levels for aliquots B and C were 1,599±179 U/L and 4,292±517 U/L, respectively. The correlation coefficients ($P<0.05$) between the hematologic parameters and HI in [B-A] and [C-A] data sets, in order of decreasing absolute percent difference, were as follows: mHb-calculated hemoglobin (cHb) (r=0.751 and r=0.943, respectively), Hct (r=-0.768 and r=-0.912, respectively), MCH (r=0.735 and r=0.905, respectively), MCHC (r=0.787 and r=0.850, respectively), cHb (r=-0.730 and r=-0.847, respectively), RBC count (r=-0.777 and r=-0.846, respectively), MCHC-cell hemoglobin concentration mean (CHCM) (r=0.766 and r=0.842, respectively), reticulocyte number (RETIC-N; r=-0.633 and r=-0.367, respectively), and MCV (r=-0.573 and r=-0.415, respectively; Table 1).

The data ranges of MCHC, RBC ghosts, mHb-cHb, and MCHC-CHCM in hemolyzed specimens showed minimum overlap with those in non-hemolyzed specimens (Table 2).

The hematologic parameters with normality and mean values with a significant difference between the [B-A] and [C-A] data sets, in order of decreasing absolute percent difference, were as follows: mHb-cHb (300% and 952%, respectively), MCH (4.1% and 18.4%, respectively), Hct (-5.2% and -16.9%, respectively), RBC count (-2.9% and -14.5%, respectively), cHb (-2.1% and -14.2%, respectively), mononuclear cell percentage (MN%, -6.4% and -12.8%, respectively), polymorphonuclear cell percentage (PMN%, 3.8% and 6.6%, respectively), and white blood cell perox (WBCP) count (-3.5% and -5.7%, respectively; Table 3).

The MCHC, RBC ghosts, mHb-cHb, and MCHC-CHCM were finally selected for including in the scoring system for detecting IVH. The scores for the 4 parameters were defined as follows: score 0<34.0, 34.0 ≤score 1 ≤36.2, 36.3 ≤score 2 ≤38.4, and score 3 ≥38.5 for MCHC (g/dL); score 0<0.02, 0.02 ≤score 1<0.03, 0.03 ≤score 2<0.04, and score 3 ≥0.04 for RBC ghosts (10^{12}/L); score 0<0.13, 0.13 ≤score 1 ≤0.38, 0.39 ≤score 2 ≤1.30, and score 3 ≥1.31 for mHb-cHb (g/dL); score 0<0.26; 0.26 ≤score 1 ≤0.95, 0.96 ≤score 2 ≤3.34, and

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Table 1. Correlation of objective hemolysis and hematologic parameters

Hematologic parameters (unit)	Hemolytic index*				Lactate dehydrogenase†			
	[B-A]‡		[C-A]§		[B-A]‡		[C-A]§	
	r	*P*	r	*P*	r	*P*	r	*P*
WBC (10^9/L)	-0.279	NS	-0.005	NS	-0.280	NS	-0.250	NS
RBC (10^{12}/L)	-0.777	<0.001	-0.846	<0.001	-0.791	<0.001	-0.655	<0.001
mHb (g/dL)	-0.337	0.041	-0.074	NS	-0.352	0.033	-0.089	NS
Hct (%)	-0.768	<0.001	-0.912	<0.001	-0.791	<0.001	-0.712	<0.001
MCV (fL)	-0.573	<0.001	-0.415	0.012	-0.585	<0.001	-0.415	0.012
MCH (pg)	0.735	<0.001	0.905	<0.001	0.761	<0.001	0.657	<0.001
MCHC (g/dL)	0.787	<0.001	0.850	<0.001	0.803	<0.001	0.635	<0.001
CHCM (g/dL)	0.203	NS	0.099	NS	0.207	NS	0.136	NS
CH (pg)	0.014	NS	-0.258	NS	0.012	NS	-0.101	NS
RDW (%)	-0.082	NS	0.141	NS	-0.071	NS	0.005	NS
HDW (g/dL)	-0.098	NS	0.316	NS	-0.091	NS	0.091	NS
PLT (10^9/L)	0.073	NS	0.247	NS	0.071	NS	0.139	NS
MPV (fL)	-0.260	NS	0.133	NS	-0.255	NS	0.005	NS
Hyper (%)	0.226	NS	0.128	NS	0.246	NS	0.142	NS
Hypo (%)	0.057	NS	0.197	NS	0.043	NS	0.137	NS
Macro (%)	-0.369	0.025	-0.166	NS	-0.395	0.016	-0.134	NS
Micro (%)	-0.051	NS	0.257	NS	-0.045	NS	0.114	NS
RBC Fragments (10^{12}/L)	-0.091	NS	0.212	NS	-0.089	NS	0.169	NS
RBC Ghosts (10^{12}/L)	0.319	NS	0.606	<0.001	0.341	0.039	0.444	0.006
MN (%)	-0.262	NS	-0.445	0.006	-0.252	NS	-0.461	0.004
PMN (%)	0.302	NS	0.431	0.008	0.293	NS	0.478	0.003
CHb (g/dL)	-0.730	<0.001	-0.847	<0.001	-0.747	<0.001	-0.648	<0.001
NEUT (%)	-0.111	NS	-0.040	NS	-0.104	NS	0.036	NS
LYMPH (%)	0.251	NS	0.021	NS	0.248	NS	0.020	NS
MONO (%)	-0.050	NS	-0.297	NS	-0.043	NS	-0.353	0.032
EOS (%)	-0.096	NS	0.278	NS	-0.110	NS	0.263	NS
BASO (%)	-0.147	NS	0.036	NS	-0.147	NS	-0.085	NS
LUC (%)	-0.128	NS	0.325	0.050	-0.132	NS	0.359	0.029
LI	0.049	NS	-0.212	NS	0.053	NS	-0.175	NS
MPXI	-0.210	NS	0.201	NS	-0.204	NS	0.061	NS
WBCP (10^9/L)	-0.468	0.005	-0.258	NS	-0.480	0.004	-0.596	<0.001
RETIC-P (%)	-0.336	NS	-0.139	NS	-0.343	NS	-0.118	NS
RETIC-N	-0.633	<0.001	-0.367	0.039	-0.634	<0.001	-0.350	0.049
CHr (pg)	0.113	NS	-0.237	NS	0.107	NS	-0.235	NS
CHm (pg)	0.123	NS	-0.277	NS	0.122	NS	-0.269	NS
mHb-cHb (g/dL)	0.751	<0.001	0.943	<0.001	0.762	<0.001	0.710	<0.001
MCHC-CHCM (g/dL)	0.766	<0.001	0.842	<0.001	0.782	<0.001	0.620	<0.001

*Mean±SD values in aliquots B and C were 1,121±147 and 3,298±328, respectively; †Mean±SD values in aliquots B and C were 1,599±179 U/L and 4,292±517 U/L, respectively; ‡[B-A] is defined as the data set obtained by subtracting the results of aliquot A from those of aliquot B; §[C-A] is defined as the data set obtained by subtracting the results of aliquot A from those of aliquot C.

Abbreviations: r, correlation coefficient; *P*, *P* value; WBC, white blood cell; NS, not significant; RBC, red blood cell; mHb, hemoglobin concentration measured by the colorimetric method; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; CHCM, cell hemoglobin concentration mean; CH, cellular hemoglobin content (mean of RBC cellular hemoglobin histogram); RDW, red cell distribution width; HDW, hemoglobin distribution width; PLT, platelet; MPV, mean platelet volume; Hyper, hyperchromia; Hypo, hypochromia; Macro, macrocytosis; Micro, microcytosis; MN, mononuclear cell; PMN, polymorphonuclear cell; cHb, calculated hemoglobin (CHCM×RBC×MCV÷1,000); NEUT, neutrophil; LYMPH, lymphocyte; MONO, monocyte; EOS, eosinophil; BASO, basophil; LUC, large unstained cell; LI, lobularity index; MPXI, myeloperoxidase index; WBCP, white blood cell count by peroxidase; RETIC-P, reticulocyte percent; RETIC-N, reticulocyte number; CHr, cellular hemoglobin mean of reticulocyte; CHm, cellular hemoglobin mean of mature RBC; mHb-cHb, mHb minus cHb; MCHC-CHCM, MCHC minus CHCM.

Table 2. Data of hemolyzed and non-hemolyzed specimens by hematologic parameters

Hematologic parameters (unit)	Data of hemolyzed and non-hemolyzed specimens							
	Aliquot A		Aliquot B			Aliquot C		
	Mean ± SD	Range	Mean ± SD	Range	N (%) of overlap*	Mean ± SD	Range	N (%) of overlap*
WBC (10^9/L)	5.01 ± 1.38	2.86-9.67	4.84 ± 1.20	2.99-8.68	37 (100)	5.06 ± 1.27	3.00-9.08	37 (100)
RBC (10^{12}/L)	4.82 ± 0.59	3.34-5.89	4.68 ± 0.62	2.90-5.90	36 (97.3)	4.12 ± 0.79	2.50-5.56	28 (75.7)
mHb (g/dL)	14.84 ± 1.59	11.6-18.2	14.95 ± 1.63	11.5-17.9	36 (97.3)	14.71 ± 1.62	11.7-17.8	37 (100)
Hct (%)	44.18 ± 4.80	33.4-53.1	41.89 ± 5.14	28.3-51.6	36 (97.3)	36.72 ± 6.78	23.9-47.1	25 (67.6)
MCV (fL)	91.84 ± 3.78	84.2-99.8	89.88 ± 3.54	83.3-96.4	36 (97.3)	89.38 ± 3.37	83.4-95.6	35 (94.6)
MCH (pg)	30.86 ± 1.30	28.4-34.6	32.13 ± 2.06	29.2-38.9	33 (89.2)	36.54 ± 5.10	29.0-48.5	17 (45.9)
MCHC (g/dL)	33.63 ± 0.67	32.3-35.3	35.67 ± 1.53	33.9-40.5	20 (54.1)	40.30 ± 5.29	34.6-57.2	7 (18.9)
		33.4-33.9†		35.2-36.2†			38.5-42.1†	
CHCM (g/dL)	34.08 ± 0.76	32.6-35.7	35.15 ± 0.75	33.9-36.6	26 (70.3)	35.19 ± 0.70	34.2-36.6	27 (73.0)
CH (pg)	31.12 ± 1.18	28.9-34.2	31.44 ± 1.23	29.0-35.0	35 (94.6)	31.30 ± 1.23	28.9-34.8	36 (97.3)
RDW (%)	12.61 ± 1.80	2.50-14.1	12.33 ± 1.73	2.60-13.7	37 (100)	12.34 ± 1.71	2.70-13.9	37 (100)
HDW (g/dL)	2.56 ± 0.20	2.24-3.02	2.54 ± 0.19	2.20-3.07	35 (94.6)	2.57 ± 0.18	2.30-3.11	35 (94.6)
PLT (10^9/L)	241.27 ± 43.07	174-340	225.49 ± 38.33	158-319	35 (94.6)	236.49 ± 44.84	169-345	35 (94.6)
MPV (fL)	8.28 ± 1.11	6.50-11.1	8.41 ± 0.76	6.90-10.0	37 (100)	8.51 ± 0.68	7.20-10.0	37 (100)
Hyper (%)	1.03 ± 0.65	0.30-3.10	1.90 ± 1.27	0.50-5.80	32 (86.5)	1.99 ± 1.27	0.60-6.30	32 (86.5)
Hypo (%)	0.61 ± 0.55	0.10-2.10	0.22 ± 0.15	0.10-0.60	37 (100)	0.24 ± 0.18	0.10-0.80	37 (100)
Macro (%)	1.18 ± 1.09	0.20-5.60	0.64 ± 0.64	0.10-2.90	32 (86.5)	0.54 ± 0.52	0.10-2.50	32 (86.5)
Micro (%)	0.37 ± 0.22	0.10-1.40	0.44 ± 0.25	0.10-1.40	37 (100)	0.48 ± 0.23	0.20-1.30	37 (100)
RBC Fragments (10^{12}/L)	0.01 ± 0.00	0.01-0.02	0.01 ± 0.00	0.01-0.02	37 (100)	0.01 ± 0.00	0.01-0.02	37 (100)
RBC Ghosts (10^{12}/L)	0.01 ± 0.00	0.00-0.02	0.02 ± 0.01	0.01-0.04	34 (91.9)	0.03 ± 0.01	0.01-0.06	14 (37.8)
		0.01-0.01†		0.02-0.02†			0.02-0.03†	
MN (%)	38.44 ± 8.08	17.1-51.9	36.01 ± 7.39	16.7-48.0	36 (97.3)	33.65 ± 7.98	19.5-52.3	36 (97.3)
PMN (%)	60.65 ± 7.80	46.8-82.0	63.00 ± 7.36	51.0-82.7	36 (97.3)	64.64 ± 7.94	46.5-79.5	36 (97.3)
cHb (g/dL)	15.05 ± 1.71	11.4-18.5	14.74 ± 1.80	10.3-18.2	36 (97.3)	12.91 ± 2.40	8.7-16.6	25 (67.6)
NEUT (%)	55.00 ± 8.07	41.3-73.1	56.18 ± 8.07	42.8-75.3	35 (94.6)	56.33 ± 8.12	40.9-76.1	35 (94.6)
LYMPH (%)	33.26 ± 8.11	11.4-48.1	33.06 ± 7.53	15.9-49.2	36 (97.3)	32.65 ± 7.45	14.9-46.1	37 (100)
MONO (%)	5.32 ± 1.28	2.80-8.80	5.34 ± 1.32	4.00-9.00	35 (94.6)	5.51 ± 1.19	3.50-8.80	37 (100)
EOS (%)	2.70 ± 1.99	0.20-9.60	2.84 ± 1.91	0.50-8.60	35 (94.6)	2.67 ± 1.80	0.30-8.20	37 (100)
BASO (%)	0.65 ± 0.31	0.20-1.80	0.51 ± 0.19	0.20-1.00	35 (94.6)	0.64 ± 0.26	0.20-1.20	37 (100)
LUC (%)	2.22 ± 0.63	1.10-4.10	2.08 ± 0.65	1.10-3.40	35 (94.6)	2.20 ± 0.63	1.20-4.10	37 (100)
LI	2.16 ± 0.10	1.93-2.33	2.16 ± 0.10	1.81-2.32	36 (97.3)	2.13 ± 0.11	1.70-2.31	36 (97.3)
MPXI	-2.16 ± 3.18	-8.0-4.0	-3.58 ± 3.24	-11.0-4.0	35 (94.6)	-3.92 ± 2.90	-12.0-1.3	35 (94.6)
WBCP (10^9/L)	5.11 ± 1.45	2.80-10.3	4.85 ± 1.30	2.82-8.81	35 (94.6)	4.82 ± 1.21	2.82-8.50	37 (100)
RETIC-P (%)	1.64 ± 0.39	0.84-2.45	1.42 ± 0.33	0.75-2.52	35 (94.6)	1.40 ± 0.32	0.87-2.32	36 (97.3)
RETIC-N	79.75 ± 21.99	44.2-131.6	67.04 ± 19.30	39.0-130.2	32 (86.5)	58.00 ± 19.78	27.3-117.8	28 (75.7)
CHr (pg)	32.09 ± 1.66	9.40-26.6	33.01 ± 1.27	31.1-36.6	35 (94.6)	32.80 ± 1.55	28.0-36.9	36 (97.3)
CHm (pg)	30.53 ± 1.28	27.8-34.0	31.24 ± 1.19	28.8-34.7	36 (97.3)	31.14 ± 1.23	29.0-34.0	36 (97.3)
mHb-cHb (g/dL)	-0.21 ± 0.27	-0.80-0.50	0.21 ± 0.51	-0.60-1.70	31 (83.8)	1.79 ± 1.47	-0.40-5.80	9 (24.3)
		-0.30--0.12†		0.04-0.38†			1.31-2.28†	
MCHC-CHCM (g/dL)	-0.45 ± 0.59	-1.40-1.60	0.51 ± 1.30	-1.40-4.00	32 (86.5)	5.10 ± 5.27	-1.00-21.9	14 (37.8)
		-0.65--0.25†		0.08-0.95†			3.35-6.86†	

*Overlap refers to the number (%) of samples lying within the range of the aliquot A group; †95% confidence interval.

Abbreviations: WBC, white blood cell; RBC, red blood cell; mHb, hemoglobin concentration measured by the colorimetric method; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; CHCM, cell hemoglobin concentration mean; CH, cellular hemoglobin content (mean of RBC cellular hemoglobin histogram); RDW, red cell distribution width; HDW, hemoglobin distribution width; PLT, platelet; MPV, mean platelet volume; Hyper, hyperchromia; Hypo, hypochromia; Macro, macrocytosis; Micro, microcytosis; MN, mononuclear cell; PMN, polymorphonuclear cell; cHb, calculated hemoglobin (CHCM × RBC × MCV ÷ 1,000); NEUT, neutrophil; LYMPH, lymphocyte; MONO, monocyte; EOS, eosinophil; BASO, basophil; LUC, large unstained cell; LI, lobularity index; MPXI, myeloperoxidase index; WBCP, white blood cell count by peroxidase; RETIC-P, reticulocyte percent; RETIC-N, reticulocyte number; CHr, cellular hemoglobin mean of reticulocyte; CHm, cellular hemoglobin mean of mature RBC.

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Table 3. Statistical data of hematologic parameters between hemolyzed and non-hemolyzed specimens

Hematologic parameters (unit)	*P* value by Kolmogorov-Smirnov test		Comparison of mean by paired t-test					
			[B - A]			[C - A]		
	[B - A]*	[C - A]†	Mean	SD	*P*	Mean	SD	*P*
WBC (10^9/L)	0.102	0.200	-0.17	0.35	0.006	0.05	0.23	0.185
RBC (10^{12}/L)	0.175	0.200	-0.14	0.19	<0.001	-0.70	0.51	<0.001
mHb (g/dL)	0.051	0.006	0.11	0.39	0.111	-0.14	0.53	0.121
Hct (%)	0.129	0.200	-2.29	2.04	<0.001	-7.46	4.57	<0.001
MCV (fL)	0.063	0.016	-1.91	0.70	<0.001	-2.41	1.54	<0.001
MCH (pg)	0.081	0.146	1.27	1.19	<0.001	5.68	4.55	<0.001
MCHC (g/dL)	0.050	0.037	2.04	1.33	<0.001	6.67	5.24	<0.001
CHCM (g/dL)	0.001	0.030	1.08	0.55	<0.001	1.11	0.76	<0.001
CH (pg)	<0.001	<0.001	0.32	0.53	0.001	0.18	0.49	0.028
RDW (%)	0.001	0.001	-0.29	0.43	<0.001	-0.27	0.46	0.001
HDW (g/dL)	<0.001	0.032	-0.01	0.12	0.448	0.01	0.16	0.761
PLT (10^9/L)	0.200	0.146	-15.78	13.32	<0.001	-4.78	22.99	0.214
MPV (fL)	<0.001	0.008	0.12	0.88	0.396	0.23	0.99	0.172
Hyper (%)	<0.001	<0.001	0.87	0.82	<0.001	0.96	1.08	<0.001
Hypo (%)	0.001	<0.001	-0.39	0.50	<0.001	-0.38	0.52	<0.001
Macro (%)	0.032	0.139	-0.54	0.52	<0.001	-0.64	0.62	<0.001
Micro (%)	<0.001	0.001	0.08	0.10	<0.001	0.11	0.11	<0.001
RBC Fragments (10^{12}/L)	<0.001	<0.001	0.00	0.00	0.003	0.00	0.00	0.017
RBC Ghosts (10^{12}/L)	<0.001	0.009	0.01	0.01	<0.001	0.02	0.01	<0.001
MN (%)	0.200	0.200	-2.44	2.27	<0.001	-4.79	5.24	<0.001
PMN (%)	0.200	0.200	2.30	2.18	<0.001	3.98	5.24	<0.001
cHb (g/dL)	0.200	0.200	-0.32	0.68	0.007	-2.14	1.70	<0.001
NEUT (%)	0.200	0.002	0.88	1.62	0.003	1.34	3.16	0.014
LYMPH (%)	0.128	0.006	-0.76	1.57	0.007	-0.61	6.32	0.560
MONO (%)	0.200	0.200	0.05	0.73	0.662	0.19	0.73	0.114
EOS (%)	0.004	0.006	0.06	0.65	0.604	-0.02	0.59	0.802
BASO (%)	0.040	0.031	-0.13	0.32	0.022	-0.02	0.36	0.786
LUC (%)	0.087	0.200	-0.10	0.47	0.202	-0.02	0.54	0.856
LI	0.200	0.200	-0.01	0.12	0.737	-0.03	0.16	0.204
MPXI	<0.001	0.023	-1.26	1.63	<0.001	-1.75	2.07	<0.001
WBCP (10^9/L)	0.200	0.143	-0.18	0.36	0.007	-0.29	0.35	<0.001
RETIC-P (%)	0.200	0.043	-0.24	0.17	<0.001	-0.22	0.35	0.001
RETIC-N	0.200	0.062	-13.7	8.79	<0.001	-19.40	22.55	<0.001
CHr (pg)	<0.001	<0.001	0.72	1.07	0.001	0.59	1.07	0.004
CHm (pg)	<0.001	<0.001	0.66	1.06	0.001	0.49	1.04	0.012
mHb-cHb (g/dL)	0.166	0.087	0.42	0.49	<0.001	2.01	1.48	<0.001
MCHC-CHCM (g/dL)	0.077	0.046	0.96	1.22	<0.001	5.55	5.20	<0.001

*[B-A] is defined as the data set obtained by subtracting the results of aliquot A from those of aliquot B; †[C-A] is defined as the data set obtained by subtracting the results of aliquot A from those of aliquot C.
Abbreviations: WBC, white blood cell; RBC, red blood cell; mHb, hemoglobin concentration measured by the colorimetric method; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; CHCM, cell hemoglobin concentration mean; CH, cellular hemoglobin content (mean of RBC cellular hemoglobin histogram); RDW, red cell distribution width; HDW, hemoglobin distribution width; PLT, platelet; MPV, mean platelet volume; Hyper, hyperchromia; Hypo, hypochromia; Macro, macrocytosis; Micro, microcytosis; MN, mononuclear cell; PMN, polymorphonuclear cell; cHb, calculated hemoglobin (CHCM×RBC×MCV÷1,000); NEUT, neutrophil; LYMPH, lymphocyte; MONO, monocyte; EOS, eosinophil; BASO, basophil; LUC, large unstained cell; LI, lobularity index; MPXI, myeloperoxidase index; WBCP, white blood cell count by peroxidase; RETIC-P, reticulocyte percent; RETIC-N, reticulocyte number; CHr, cellular hemoglobin mean of reticulocyte; CHm, cellular hemoglobin mean of mature RBC.

Table 4. Score ranges of hemolytic parameters between hemolyzed and non-hemolyzed specimens

Status of hemolysis	Score sum N of the four hematologic parameters*												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Non-hemolyzed	20	12	3		1†		1						
Hemolyzed (2 times‡)	4	8	1	6	3	3	4	4	1	2	1		
Hemolyzed (5 times‡)		2	1	2		1	4	4	2	2	2	7	10

*Mean cell hemoglobin concentration (g/dL, score 0 <34.0, 34.0 ≤score 1 ≤36.2, 36.3 ≤score 2 ≤38.4, score 3 ≥38.5), red blood cell ghosts (10^{12}/L, score 0 <0.02, 0.02 ≤score 1 <0.03, 0.03 ≤score 2 <0.04, score 3 ≥0.04), measured Hb-calculated Hb (g/dL, score 0 <0.13, 0.13 ≤score 1 ≤0.38, 0.39 ≤score 2 ≤1.30, score 3 ≥1.31), and mean cell hemoglobin concentration-cell hemoglobin concentration mean (g/dL, score 0 <0.26, 0.26 ≤score 1 0.95, 0.96 ≤score 2 ≤3.34, score 3 ≥3.35); †Scores of the four hematologic parameters was equally 1; ‡Aliquots B and C were mechanically hemolyzed by aspirating two and five times, respectively, through a 27-gauge needle.

score 3 ≥3.35 for MCHC-CHCM (g/dL). At a cutoff value of 3 for the hemolysis score (defined as the sum of all the scores of the 4 parameters), the IVH of aliquots B and C were detected as 64.9% and 91.9%, respectively (Table 4).

This new scoring system was established in January 2013 at a tertiary-care university hospital in Wonju, Korea.

DISCUSSION

To accurately detect IVH with hematology analyzers, it is necessary to measure only the cell-free Hb in the blood specimens without adding RBC lysing solution. However, cell-associated and cell-free Hb are not distinguishable by colorimetric methods, which are used by hematology analyzers [5]. In the Advia hematology analyzer, Hb is evaluated by using two methods: (1) a standard cyanmethemoglobin colorimetric method and (2) flow cytometry. Flow cytometry is based on low- and high-angle laser light scatter and is used for measuring the volume of individual RBCs and Hb concentration [6]. The low- and high-angle light scatter signals are transformed into RBC volume and Hb concentration values, respectively. Flow cytometry enables the determination of the actual Hb mass within the RBC by multiplying the RBC volume by the Hb concentration on a cell-by-cell basis; therefore, this method can possibly be used for differentiating cell-associated and cell-free Hb. A novel analyte, designated as CHCM, has been introduced for the Advia hematology analyzer system. The CHCM indicates the average concentration of Hb within individual RBCs as measured by flow cytometry [6].

In comparison, the MCHC is a calculated value that indicates the average Hb concentration within RBCs based on an analysis of total colorimetric Hb in a hemolyzed specimen. Therefore, hemolysis or lipemia may result in a falsely elevated MCHC, whereas the CHCM is not affected. Among the hematologic parameters, the MCHC data range in hemolyzed specimens had the least overlap compared with non-hemolyzed specimens (Table 2). However, the mean CHCM in hemolyzed specimens was higher than that in non-hemolyzed specimens, although [B-A] and [C-A] showed a non-normal probability distribution and low correlation coefficients for HI values (Tables 1 and 3).

During hematologic analysis, the CHCM and MCHC are automatically compared by the Advia software, and a Comparison Error MCHC/CHCM flag is generated if they differ by >1.9 g/dL [6]. Compared to this study (Table 2), a MCHC-CHCM cutoff value of 1.9 g/dL might be capable of detecting error conditions that could be affecting one or more of the three results (i.e., RBC, MCV, or Hb), used for calculating MCHC; however, this is associated with a low sensitivity for detecting IVH. It seemed that the MCHC-CHCM range was more useful than the MCHC-CHCM cutoff value for discriminating hemolyzed and non-hemolyzed specimens because the correlation coefficients of MCHC-CHCM and HI were high and increased in proportion with the increase in hemolysis (0.766 in [B-A] and 0.842 in [C-A]).

In the Advia system, cHb values are calculated by using CHCM instead of MCHC to avoid lipemia interference [6]. In comparison to CHCM, cHb reflects the change effects of MCV and RBC by hemolysis, which might potentiate the hemolytic effect, because both MCV and RBC are decreased with hemolysis. The cHb was a suitable parameter for IVH because the mean cHb value in hemolyzed specimens was significantly lower than that in non-hemolyzed specimens, and the correlation coefficients of cHb and HI were high and proportionally decreased with hemolysis (-0.730 in [B-A] and -0.847 in [C-A]). However, 97.3% and 67.6% of cHb data in aliquots B and C, respectively, were overlapped with those in non-hemolyzed specimens (Table 2). Among the hematologic parameters, mHb-cHb showed the highest correlation coefficients with HI and the second lowest overlap between hemolyzed and non-hemolyzed specimens (Tables 1 and 2).

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RBC ghosts, one of the hematologic flags, are displayed on the PLT scatter cytogram of the Advia system. The PLT scatter cytogram is the graphical representation of two light-scatter measurements: (1) the high-angle light scatter plotted on the X-axis and (2) the low-angle light scatter plotted on the Y-axis. The low- and high-angle light scatter signals are transformed into volume and refractive index (n) values, respectively [6]. The RBC ghost morphology flag is generated, if the number of events in the RBC ghost area of the PLT scatter cytogram is $>10^{11}$ cells/L. The events counted in the RBC ghost area of the PLT scatter cytogram have refractive indices <1.350. The sample-related causes of RBC ghost morphology flag are the presence of hemolysis, cryoglobulins, chylomicrons, pyropoikilocytosis, and lipemia [6, 7]. In the present study, the number of events in the RBC ghost area could be used as an informative marker of IVH, even though it is lower than the cutoff value of flag generation, regardless of the severity of hemolysis. This may be because the methods for measuring RBC ghosts is different from the detection method for Hb, and the overlap percentage of RBC ghost between aliquots A and C was as low as 37.8%. Finally, the four hematologic parameters and their scores are displayed on a workstation monitor for laboratory personnel, and the sum of scores is reported through a personal computer monitor to the medical staff. Our scoring system will provide an effective screening method for detecting IVH, although the associated sensitivity is not quite satisfactory. In addition, the scoring system will educate clinicians and nurses about the relationship between improper blood sampling and hematologic testing results.

One limitation of our scoring system was that we did not evaluate the relationship between other preanalytic interferents, such as lipemia and icterus, and the four hematologic parameters. Additionally, the HI and LDH levels were not measured in the plasma of aliquot A. If any of the aliquot A sample had IVH, it could possibly influence the data analysis of hemolytic parameters. We expect that a more sensitive and precise scoring system for IVH, according to age, Hb concentration, and underlying hematologic disease, will be examined in future studies.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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