Research Article [Araştırma Makalesi]

Effects of hemolysis on the assays of serum CK, CK-MB activities and CK-MB (mass), troponin and myoglobin measurements

[Serum CK ve CK-MB aktiviteleri ile CK-MB kütle, troponin ve miyoglobin ölçümleri üzerine hemolizin etkisi]

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ABSTRACT

Aim: The aim of the study was to investigate the effects of in vitro hemolysis on serum creatine kinase and creatine kinase MB isoenzyme activities with creatine kinase MB mass, troponin I and myoglobin measurements.

Materials and methods: We prepared serum pools having analyte concentrations at normal and pathological values. Hemolysate was added into serum pools to obtain aliquotes with final hemoglobin concentrations of 21, 10.5, 5.25, 2.625, 1.312, 0.656, 0.328, 0.164, 0.08 and 0.041 g/L. Creatine kinase (by **N-acetylcysteine-activated IFCC method**) and creatine kinase MB activities (by immunoinhibition method) were measured in these pools. Creatine kinase MB (mass), troponin I and myoglobin concentrations were measured by chemiluminescence method. Mean percent changes were calculated and graphed as interferographs.

Results: It was found that the positive interference due to hemolysis began to exceed the limit of 10% at lower Hb concentration for CK-MB activity than CK activity. When reference change value were considered, the critical effect of hemolysis began at higher hemoglobin concentrations. Hemolysis did not affect creatine kinase MB mass, troponin and myoglobin measurements when the limit of 10% change was considered.

Conclusions: CK-MB activity is affected more profoundly than CK activity by hemolysis. Whereas CK-MB (mass), troponin I and myoglobin are absolutely not affected by hemolysis.

Key Words: Hemolysis, interference, creatine kinase MB

Conflict of Interest: Authors do not have any conflict of interest.

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ÖZET

Amaç: Çalışmanın amacı, kreatin kinaz (CK) ve kreatin kinaz MB (CK-MB) aktivitesi ile CK-MB (kütle), Troponin I ve Miyoglobin ölçümleri üzerine hemoliz etkisinin incelenmesidir.

Gereç ve yöntemler: Çalışmada, normal ve patolojik değerde analit konsantrasyonuna sahip serum havuzları hazırlandı. Bu havuzlara maksimum hemoglobin konsantrasyonu 21 g/L olacak şekilde hemolizat eklendi. Seri seyreltimlerle hemoglobin konsantrasyonu 21, 10.5, 5.25, 2.625, 1.312, 0.656, 0.328, 0.164, 0.08 ve 0.041 g/L olan serum havuzları elde edildi. Bu havuzlarda kreatin kinaz aktivitesi IFCCyöntemi ile, CK-MB aktivitesi immünoinhibisyon yöntemi il ölçüldü. Kreatin kinaz (kütle), troponin I ve miyoglobin ölçümleri kemiluminesan yöntemle yapıldı. Hemoliz etkisine bağlı ortalama % değişim hesaplandı ve interferograf olarak sunuldu.

Bulgular: Hemolize bağlı pozitif interferans %10 sınırı esas alındığında CK-MB aktivitesi için CK aktivitesine göre daha düşük hemoglobin konsantrasyonlarında başladı. Referans değişim değeri göz önüne alındığında hemoliz etkisi daha yüksek hemoglobin konsantrasyonlarında başladı. Hemoliz, %10 sınırları esas alındığında CK-MB (kütle), troponin I ve miyoglobin ölçümlerini etkilemedi.

Sonuç: CK-MB aktivitesi hemolizden CK aktivitesine göre daha çok etkilenmektedir. CK-MB (kütle), troponin I ve miyoglobin ölçümleri ise hemolizden etkilenmemektedir.

Anahtar kelimeler: Hemoliz, interferans, kreatin kinaz MB

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

The diagnostic importance of the measurement of serum creatine kinase MB isoenzyme activity or mass concentration as well as cardiac troponin and myoglobin in acute coronary syndrome is well known and widely used in clinical laboratories. Although CK total and CK-MB isoenzyme activities are not recommended in "post troponin era", measurement of these activities is an acceptable alternative in institutions where cardiac troponin (cTn) or CK-MB mass are not available [1].

In vitro hemolysis is recognised as a frequent source of error in clinical laboratory and the measurement of CK and CK-MB activity is affected positively because enzymes and intermediates [adenylate kinase (AK), ATP, glucose-6-phosphate (G6P)] liberated from erythrocytes may affect the lag phase and the side reactions occurring in the assay system [2].

In contrast to immunoinhibition, immunoassays measure CK-MB mass concentrations in which two antibodies having affinity for different parts of the CK-MB dimer used. Mass assays based on sandwich techniques are more sensitive than activity-based methods [3,4].

The effect of hemolysis on the measurement of CK-MB activitiy has been described in the literature previously but there are few studies analyzing the degree of this effect and there are not sufficient studies investigating the relationship between the degree of the hemolysis and the clinical decision point [5,6].

Cardiac troponins are presently regarded as the most cardiac-specific of currently available biochemical markers and myoglobin is the earliest marker for the diagnosis of myocardial injury. Several commercially available immunoassays measure the concentration of troponin I and myoglobin in serum. Hemolysis effect on troponin and myoglobin assays have been also evaluated by many investigators but there are still discrepancies about the level of this interference [7,8].

In the present study we aimed to evaluate the effect of *in vitro* hemolysis on the measurement of CK, CK-MB activity, CK-MB mass, troponin and myoglobin and to explore where this interference could affect the clinical decision point and also to investigate where this interference could affect the clinical decision point.

For this reason, we used the limit of 10% for the mean percent changes and reference change values as the clinical decision point. To the best of our knowledge, this type of comparison has not yet been performed for these analytes.

Materıals And Methods

Preparation of hemolysates

Hemolysates were prepared by a modification of a method described previously as follows [9]. Two-mL of fresh whole-blood sample with EDTA (hemoglobin values >16 mg/dL) was used. After centrifugation at 1500 x g for

5 min, plasma was removed and discarded. Ten – mL of 0.15 mol/L sodium chloride was added and mixed by inversion. Erythrocyte suspansion was recentrifuged at 1500 x g for 5 min and the supernatant (saline) was excluded. This process was repeated three times. After removing of saline, the erythrocyte pellet was stored (without adding deionised water) at – 80 °C. After thawing at room temperature, lysate was recentrifuged at 10 000 x g for 5 minute and supernatant removed. After measuring its hemoglobin (Hb) value by Beckman Coulter Gen-S analyzer (Beckman-Coulter Inc., Fullerton, CA, USA) (30 g/L), the hemolysate was aliquoted for study.

Preparation of normal and pathologic serum pools

Fourty - mL of normal serum pool was prepared from clear, visibly non-hemolysed patients' sera. Cardiac biomarkers of these sera were within reference limits (CK-MB activity <20 U/L, CK-MB mass <3.1 µg/L, CK activity <140 U/L, troponin I <0.034 µg/L and myoglobin <47.5 µg/L). Fourty - mL of pathologic serum pools was prepared from non-hemolysed sera of patients with acute coronary syndrome and cardiac biomakers were above critical values (CK-MB activity >100 U/L, CK-MB mass >10 µg/L, CK activity >1500 U/L troponin I > 0.2 µg/L and myoglobin >100 µg/L).

Addition of hemolysate to the pools

Six mL aliquot from 40 mL of normal serum pool was added into a glass test tube and labelled as "Serum pool A,. the remaining 34 mL of serum pool was labelled as "Serum pool B"; 0.42 mL of serum was removed and discarded from serum pool A and equal volume of hemolysate (30 g/L) was added to the same pool to give a final Hb concentration of 21 g/L and vortexed well.

To equalize the dilution effect of the hemolysate, 2.9 mL of serum was removed and discarded from serum pool B and equal volume of sodium chloride was added to the same pool and vortexed. Ten glass test tubes were numbered from one to ten. Serum pool A was numbered as tube 11.

Three mL aliquot from serum pool A was diluted with equal volume aliquot from serum pool B in another test tube (number ten) and vortexed well. The calculated final Hb concentration of second test tube was half of the serum pool A because of the two-fold dilution. These serial dilutions were repeated for other tubes (except the last one) to give final Hb concentrations of 21, 10.5, 5.25, 2.625, 1.312, 0.656, 0.328, 0.164, 0.08, 0.041 g/L. Only 3 mL aliquot from serum pool B was added to the last tube (number one). Hb concentration of serum pool B was considered as 0.0 g/L. Same procedure was applied to the pathological serum pool.

Measurement of analytes

Hemolysis was assayed by the spectrophotometric oxy-hemoglobin method at 415 nm as described previously

(Shimadzu spectrophotometer, Shimadzu Corporation, Tokyo, Japan) [10]. Dilution was performed for Hb values above 2 g/L. Samples which Hb values greater than 10 g/L were measured by Beckman Coulter analyzer. CK-Olympus activity (code OSR6221, original Olympus kit) were assayed with N-acetylcysteine-activated IFCC method on Olympus AU640 analyzer (Beckman Coulter International-Mihsima Olympus Co. Ltd. Japan). CK-MB Olympus activity (code OSR61155, original Olympus kit) was assayed with immunoinhibition method in Olympus AU640.

CK-Roche activity (code 12132672 216, original Roche kit) was assayed with N-acetylcysteine-activated IFCC method and applicated to the Olympus AU640 analyzer. CK-MB Roche activity (code 12132893 216, original Roche kit) were assayed with immunoinhibition method and applicated to the Olympus AU640 autoanalyzer. Troponin I (LIAISON^R Ref 315101), Myoglobin (LIAISON^R Ref 315301), CK-MB mass (LIAISON^R Ref 31520) were assayed by LIAISON analyzer (DiaSorin, s.p.A, Saluggia, Italy). Lipemia-Icter-Hemolysis index (LIH Index) of Olympus 640 were also recorded during the analyses. All the analyses were performed in triplicate.

Calibrators and calibration

Original Olympus system calibrator and CK-MB calibrator (no 66300, CK value: 155U/L, no ODR30034 CK-MB value: 140 U/L, traceable to the IFCC reference method), Roche calibrators CFAS, CFAS CK-MB (no 171684, CK value: 304 U/L, no 11447394, CK-MB value: 127 U/L, traceable to the IFCC reference method), LIAISON^R Troponin I Cal Low and High (Ref 319118), original myoglobin kit calibrator 1 and 2 (Ref 315301), LIAISON^R CKMB Cal Low and High (Ref 319125 in data sheet: they are standardised against "in-house" reference calibrator) were used for calibration. Additionally, activity calculations based on molar absorptivity coefficient were performed for enzyme activity measurements.

Precision

Within-run precision: Both normal and pathologic serum pools were analyzed for 21 consecutive replicates (before hemolysate addition) in the same run.

Between-run precision: both pools were aliquoted and stored at -20 °C and assayed in 20 different days.

Referance change values (RCV): Biological variation data was taken from the Biological Variation Database in the Westgard Website [11].

Within-run CV, (CV_{WR}) and beetwen-run CV, (CV_{BR}) were used for calculation of total analytical variation.

RCV was calculated as follows;

$$RCV = \sqrt{2} \times z \times \sqrt{(CV_A^2 + CV_I^2)}$$

CV_A: Total analytical variation

$$CV_A = \sqrt{(CV_{WR}^2 + CV_{BR}^2)}$$

CV_I: intraindividual biological variation

z score: 1.96 (for probability of 95%)

Hemolysis interference was expressed as mean percent change (MPC). MPC was calculated as follows;

$$F = [(C - C_0)/ C_0] \times 100$$

F = mean percent change

C = analyte concentration in sample with hemolysate

C_0 = analyte concentration in sample without hemolysate

Results

Basal Hb levels determined spectrophotometrically were 0.05 g/L for normal serum pool and 0.06 g/L for pathologic serum pool. Final Hb concentration of last tube (tube no 11) was 21 g/L. Measured Hb levels compared with those calculated ones and LIH index of Olympus analyzer were shown in Table 2 and 3. Concentrations and MPC for all anlaytes were shown in the same tables with corresponding Hb levels. Positive interference due to hemolysis was observed for the activity assays for both serum pools. This effect began to exceed the limit of 10% at Hb concentration of 1 g/L for CK-Roche and 4 g/L for CK-Olympus in normal serum pool and at Hb level above 5 g/L for CK-Roche and Hb level above 14 g/L for CK-Olympus in pathologic serum pool. Results were similar for CK-MB activity assays. The limit of 10% as critical point was exceeded at Hb level above 0.37 g/L for both CK-MB Olympus and Roche. But MPC for CK-MB Roche in normal serum pool was greater than those for CK-MB Olympus at this Hb level (21.3% and 16.2%, respectively). In the pathologic pool, MPC began to exceed the limit of 10% at a Hb level above 0.7 g/L for CK-MB Roche and at a Hb level above 0.8 g/L for CK-MB Olympus (Table 2 and 3). We also showed the interference effect with interferographs (Figure 2 and 3). Roche activity assays began to be affected at lower Hb concentration than Olympus for both CK and CK-MB. This difference was greatest at final Hb concenterion of 21 g /L.

In addition, we evaluated the hemolysis effect based on RCV for CK and CK-MB activity (Table 4). There were not observed any value exceeded the limit of RCV for CK (Olympus and Roche) in normal pool. MPC for only CK-Roche in pathologic serum pool exceeded the RCV at Hb level of 5.5g/L. In normal serum pool MPC for CK-MB exceeded the limit of RCV at Hb of 1.35 g/L and at 5g/L for pathologic serum pool for both commercial kits. (RCV in normal and pathologic serum pools, in turn, 58.1% for Olympus, 57.1% for Roche and 77.6% for Olympus, 98.8% for Roche). In creatine kinase MB mass, troponin and myoglobin assays, there were not observed any hemolysis effect exceeded the limit of 10% or of RCV at any level of Hb.

We also calculated the calibrator values of CK and CK-MB based on molar absorbtivity and compared those of manufacturer determined (Table 5). Calculated calibra-

Table 1. Mean analyte concentration (m), standart deviation (SD) and within-laboratory coefficient of variation (CV, %) in normal and pathologic serum pools.

Analytes	Normal Serum Pool			Pathologic Serum Pool		
	m	SD	CV, %	m	SD	CV, %
CK activity, U/L						
Olympus	120	2.95	2.45	1449	15.8	1.1
Roche	114	1.89	1.65	1486	21	1.41
CK-MB activity U/L						
Olympus	13.1	0.7	5.39	123	1.6	1.3
Roche	12.6	0.69	5.50	119	1.6	1.4
CK-MB (mass), µg/L	1.95	0.09	4.64	91.1	1.5	1.6
Troponin, µg/L	0.017	0.003	17.6	8.60	0.26	2.97
Myoglobin, µg/L	39.9	1.50	3.75	2024	31.6	1.56

tor values were 285 U/L for CK-Roche and 142 U/L for CK-Olympus. These values are close to those of manufacturer determined (304 for CK-Roche and 155 for CK-Olympus). Whereas, the values calculated for CK-MB calibrators (CK-MB Roche and Olympus, in turn, 119 U/L and 100 U/L) were lower than those of manufacturer determined for both commercial products (CK-MB Roche and Olympus, in turn, 127 U/L and 140 U/L).

Discussion

Samples with hemolysis are common and unfavorable occurrence in laboratory practice. Olympus AU640 is equipped with automated system for semiquantitavie detection of lipemic, icteric and hemolyzed samples (LIH index). Lippi et al. compared the efficiency of different analyzers including Olympus AU680 to evaluate the hemolyzed samples and found that results were satisfactory [12]. In another study, Simundic et al. assessed the comparability of automated spectrophotometric detection by Olympus AU 2700 and visual inspection of hemolyzed samples and showed a comparable rate of detection for hemolyzed samples [13].

In the present study, Hb levels in the samples detected automatically with LIH index of Olympus AU 640 were very close to manually measured and calculated Hb values (Table 2 and 3). Hb value in tube five was 0.37 g/L for normal serum pool and was flagged as "N" by LIH index of Olympus analyzer. After this tube, hemolysis was visually detectable from the color of the serum and flagged as "+" (Figure 1). These results were consistent with the manufacturer's claim (N, if Hb value <0.5 g/L) and studies mentioned above. But in normal serum pool, at Hb level of 0.37 g/L, MPC for CK-MB activity exceeded the limit of 10% due to hemolysis interference for both commercial kits. Therefore, it can be said that LIH index of Olympus can be used to identify the inappropriate hemolyzed samples but this is not reliable enough for CK-MB activity in the samples with normal range.

It has been previously documented that adenylate kinase catalyzes the reaction; 2 ADP ⇄ AMP + ATP, and increased AK levels in hemolyzed samples leads to an apparent increase in creatine kinase activity [14,15]. It can be diminished by the addition of either AMP, which is a weak competitive inhibitor of AK, or diadenosine pentaphosphate, which is a powerful competitive inhibitor (2). To evaluate this interference, we compared the CK and CK-MB activity measured by Olympus and Roche commercial kits. We used limit of 10% change from baseline for MPC and interferographs described earlier [16-18]. We observed that in normal serum pool, MPC began to exceed the limit of 10% at lower Hb concentration for CK-Roche activity than CK Olympus. This early effect was apperent in interferographs (Figure 2-A). Similar effect was observed for the pathologic pool (Figure 2-B). Sonntag, in his study, found that the limit of 10% for CK activity was exceeded at Hb level of 2.5 g/L [19]. But CK activity level (37 U/L) in his study was lower than those of us (CK activity >100 U/L). In another study Lippi et al. used the limit of 11.5% (desirable bias) and found that hemolysis effect exceeded this limit at Hb level of 2.6 g/L for CK activity of 118 U/L (20). But they used different commercial kits and analyzers. Because of these differences between commercial products, we suggest that, when evaluating hemolysis effect for CK activity, users should be stick to limitations stated by manufacturer in kit inserts.

On the other hand, MPC of CK for both commercial kits began to exceed the limit of 10% at lower Hb levels for normal serum pool than patological serum pool. This can be explained by the different analyte concentrations in serum pools. It is well known that enzymatic assays have better precision at higher analyte concentrations. In this study, precision values were better at high analyte concentrations for both commercial kits (Table 1). Therefore, greater hemolysis interference at lower analyte concentrations was considered as an expected effect.

We also documented that CK-MB activity assays using immunoinhibition method was positively interfered with the hemolysis for both commercial kits. But CK-MB Roche assays started to be effected at lower Hb con-

Table 2. Analyte concentrations, mean percent changes (MPC), measured Hb (Hbm), calculated Hb (Hbc) and LIH index of Olympus in normal serum pool.

Mean Analyte Concentrations (C) and Mean Percent Change (MPC, %)

Tube no	Hemoglobin, g/L			CK Olympus (U/L)		CK Roche (U/L)		CK-MB Olympus (U/L)		CK-MB Roche (U/L)		CK-MB Mass (µg/L)		Troponin (µg/L)		Myoglobin (µg/L)	
	Hbm	Hbc	LIH index	C	MPC	C	MPC	C	MPC	C	MPC	C	MPC	C	MPC	C	MPC
1*	0.05	0***	N	118	0,00	113	0,00	12,3	0,00	11,9	0,00	1,88	0,00	0,013	0,00	37,27	0,00
2	0.09	0.041	N	118	-0,28	113	0,30	12,0	-2,70	11,9	-0,28	1,91	1,95	0,012	-2,63	36,17	1,95
3	0.13	0.082	N	119	0,56	116	2,66	12,3	0,00	12,0	0,56	1,78	-4,97	0,011	-10,53a	37,17	-4,97
4	0.21	0.164	N	119	0,85	114	1,51	12,7	2,70	12,6	5,60	1,94	3,55	0,015	15,79	37,50	3,55
5	0.37	0.328	N	120	1,69	117	4,00	14,3	16,2a	14,4	21,3a	1,96	4,44	0,014	13,16	38,20	4,44
6	0.7	0.656	+	121	2,26	122	8,08	15,0	21,6	16,1	35,3	1,89	0,53	0,013	5,26	36,00	0,53
7	1.35	1.312	++	124	5,08	126	12,0a	19,3	56,8	20,1	68,6b	1,85	-1,42	0,012	-7,89	37,47	-1,42
8	2.65	2.625	+++	124	5,37	144	27,9	28,3	130b	31,7	166	1,87	-0,18	0,012	-7,89	41,17	-0,18
9	5.3	5.25	abn	131	11,0a	181	60,5	41,7	238	52,6	342	1,89	0,89	0,015	15,79a	40,63	0,89
10	10.5	10.5	abn	141	19,8	255	127b	58,0	370	105	779	1,85	-1,42	0,012	-7,89	41,53	-1,42
11**	21	21	abn	154	30,5	431	283	120	873	232	1845	1,86	-0,71	0,014	13,16a	39,03	-0,71

*Serum pool B; **, serum pool A; ***Hb value was accepted as "0"; [a], MPC values that exceed the limit of 10%; [b], MPC values that exceed the RCV values, (RCV values; for CK: Olympus and Roche, in turn, 64.8% and 62.9%, for CK-MB Olympus and Roche; in turn, 58.1% and 57.1%, for troponin and myoglobin (no value exceeded); in turn, 96.4% and 39.6%).

Alarm signals for LIH index: N, Hb value <0.5 g/L.

+, ++, +++, Hb values, in turn, 0.5 - 0.9 g/L, 1.0 - 1.9 g/L and 2.0 - 2.99 g/L, Abn, Hb value >3 g/L.

Table 3. Analyte concentrations, mean percent changes (MPC), measured Hb (Hbm), calculated Hb (Hbc) and LIH index of Olympus in pathologic serum pool.

Tube no	Hemoglobin, g/L Hbm	Hemoglobin, g/L Hbc	LIH index	CK Olympus (U/L) C	CK Olympus (U/L) MPC	CK Roche (U/L) C	CK Roche (U/L) MPC	CK-MB Olympus (U/L) C	CK-MB Olympus (U/L) MPC	CK-MB Roche (U/L) C	CK-MB Roche (U/L) MPC	CK-MB Mass (µg/L) C	CK-MB Mass (µg/L) MPC	Troponin (µg/L) C	Troponin (µg/L) MPC	Myoglobin (µg/L) C	Myoglobin (µg/L) MPC
1*	0.06	0***	N	1423	0,00	1472	0,00	120	0,00	115	0,00	90,8	0,00	8,89	0,00	2034	0,00
2	0.10	0.041	N	1424	0,05	1475	0,20	121	0,28	116	1,16	91,6	-0,28	8,90	0,06	2052	0,90
3	0.14	0.082	N	1426	0,16	1474	0,14	119	-0,83	117	1,74	91,9	1,21	9,03	1,57	2034	0,00
4	0.22	0.164	N	1421	-0,19	1476	0,27	119	-0,83	117	2,33	91,2	0,44	8,93	0,39	2031	-0,13
5	0.38	0.328	N	1435	0,84	1484	0,77	127	5,82	121	5,81	90,0	-0,33	8,80	-1,01	1989	-2,18
6	0.71	0.656	+	1432	0,63	1492	1,31	130	8,31	127	10,8 [a]	90,0	1,32	8,91	0,17	2045	0,57
7	1.36	1.312	++	1447	1,66	1499	1,79	148	23,3 [a]	141	22,7	92,0	1,87	8,68	-2,42	2042	0,43
8	2.67	2.625	+++	1449	1,78	1556	5,66	167	38,8	169	47,7	91,0	0,22	8,97	0,90	2029	-0,23
9	5.3	5.25	abn	1493	4,89	1627	10,5 [a]	214	77,6 [b]	228	98,8 [b]	91,0	-0,33	8,97	0,84	2034	0,03
10	10.5	10.5	abn	1527	7,31	1778	20,8	327	172	357	211	91,8	1,05	8,94	0,51	2022	-0,56
11**	21	21	abn	1590	11,7 [a]	2057	39,7	562	367	619	440	91,8	-0,61	8,85	-0,51	2031	-0,15

Mean Analyte Concentrations (C) and Mean Percent Change (MPC, %)

*Serum pool B; ** serum pool A; ***Hb value was accepted as "0"; [a] MPC values that exceed the limit of 10%; [b] MPC values that exceed the RCV values, (RCV values; for CK: Olympus and Roche, in turn, 63.9% and 62.7%, for CK-MB Olympus and Roche; in turn, 54.3% and 54.2%, for troponin and myoglobin (no value exceeded); in turn, 41.6% and 43.1%).

Alarm signals for LIH index; N, Hb value <0.5 g/L.

+, ++, +++, Hb values, in turn, 0.5 - 0.9 g/L, 1.0 - 1.9 g/L and 2.0 - 2.99 g/L, Abn, Hb value >3 g/L.

centration than CK-MB Olympus for both serum pools (Table 2 and 3). Therefore, we can say that Olympus commercial kits have better performance than Roche in hemolyzed samples for CK and CK-MB activity.

In this study, we also observed that positive interference due to hemolysis on CK-MB activity started to increase at lower Hb values than CK activity for both commercial kits (Figure 2 E, F and Figure 3 A, B). This is an unexpected result because CK-MB activity assay uses the same enzymatic reaction with CK. Only difference is that in immunoinhibition techniques for measurement of CK-MB activity, an anti-CK-M subunit antiserum is used to inhibit both M subunits of CK-MM and the single M subunit of CK-MB. The result is multiplied by two to achieve final concentration. To determine CK-MB, this technique assumes the absence of CK-BB and other sources of interference [1]. We found no study analyzing the different effect of hemolysis on CK and CK-MB activities in literature. Yucel et al. evaluated the effect of mild hemolysis on CK-MB activities only and speculated that positive interference began at lower Hb concentrations than those of CK and increases positively with Hb concentrations. They have attributed this early effect to the lower concentration of CK-MB [6].

To equalize this concentration effect we compared CK-MB activity values in pathologic serum pool with CK activity values in normal serum pool. (in turn, CK-MB Olympus = 120 U/L, CK-Olympus = 118 U/L). But interestingly, even at this similar values, CK-MB activity in pathological serum pool started to be affected at lower Hb levels than those of CK activity in normal serum pool for both commercial kits (Table 2 and 3). Limit of 10% for CK in normal serum pool was exceeded at a Hb value of 1 g/L for CK-Roche and 4 g/L for CK-Olympus. Whereas, same limit for CK-MB activity was exceeded at Hb above 0.7 g/L for CK-MB Roche and above 0.8 g/L for CK-MB Olympus. But this effect was greater between CK-Olympus and CK-MB Olympus. Whereas, both assays using the same reaction steps had similar

concentration of adenosine monophosphate (AMP) and diadenosine pentaphosphate as inhibitors of adenylate kinase. In order to explain this difference, calculations based on molar absorbtivity were made for each calibrator of CK and CK-MB. We used the data obtained from the application parameters on the Olympus analyzer and compared the values with those stated by manufacturer in the calibrator inserts (Tablo 5). The calculated calibrator values for CK-MB were lower than those of manufacturer determined for both commercial products. The ratio between two values (determined by manufacturer / calculated) was 1.4 for Olympus and 1.1 for Roche. In this context we can speculate that if the manufacturer are using this constant to equalize the calibrator values with the reference material, this difference between activity values could be considered as reasonable. The measurement of the CK-MB activiy in the samples with normal range may not be affected by using this constant. But in hemolyzed samples, the errors coming from the interfering agents will be multiplied by this constant (in addition to constant "2" used to achieve final CK-MB activity) and largely reflected in the patient results. We also found that, CK-MB Olympus activity was affected at lower Hb values than CK-Olympus according to the Roche. This could be explained by the difference between constants, because the constant calculated for Olympus (1.4) was greater than those for Roche (1.1).

Reference change value (RCV) described by Harris and Yasaka is used to assess the clinical important change between two consecutive test results [21]. In this study we calculated RCV for all analytes to evaluate intereference effect. We found that, when RCV was used as medical decision point instead of limit of 10%, the effect of interference was began to exceed the limit of RCV at higher Hb concentration for CK and CK-MB activity in both pools (Table 2 and 3). Although our precision values for analytes evaluated in this study were within the desired range ($CV_A < 0.5\ CV_I$ for all analytes except troponin in normal serum pool) these analytes had hig-
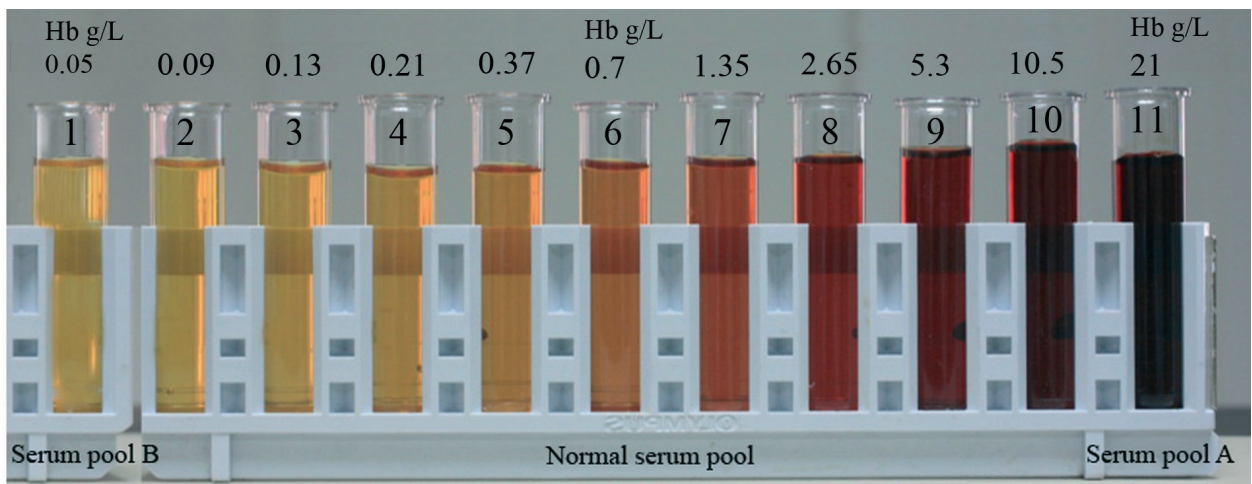

Figure 1. Visual appearance of normal serum pool. Color change due to hemolysis is started to be visible at tube 5 and apperent at tube 6.
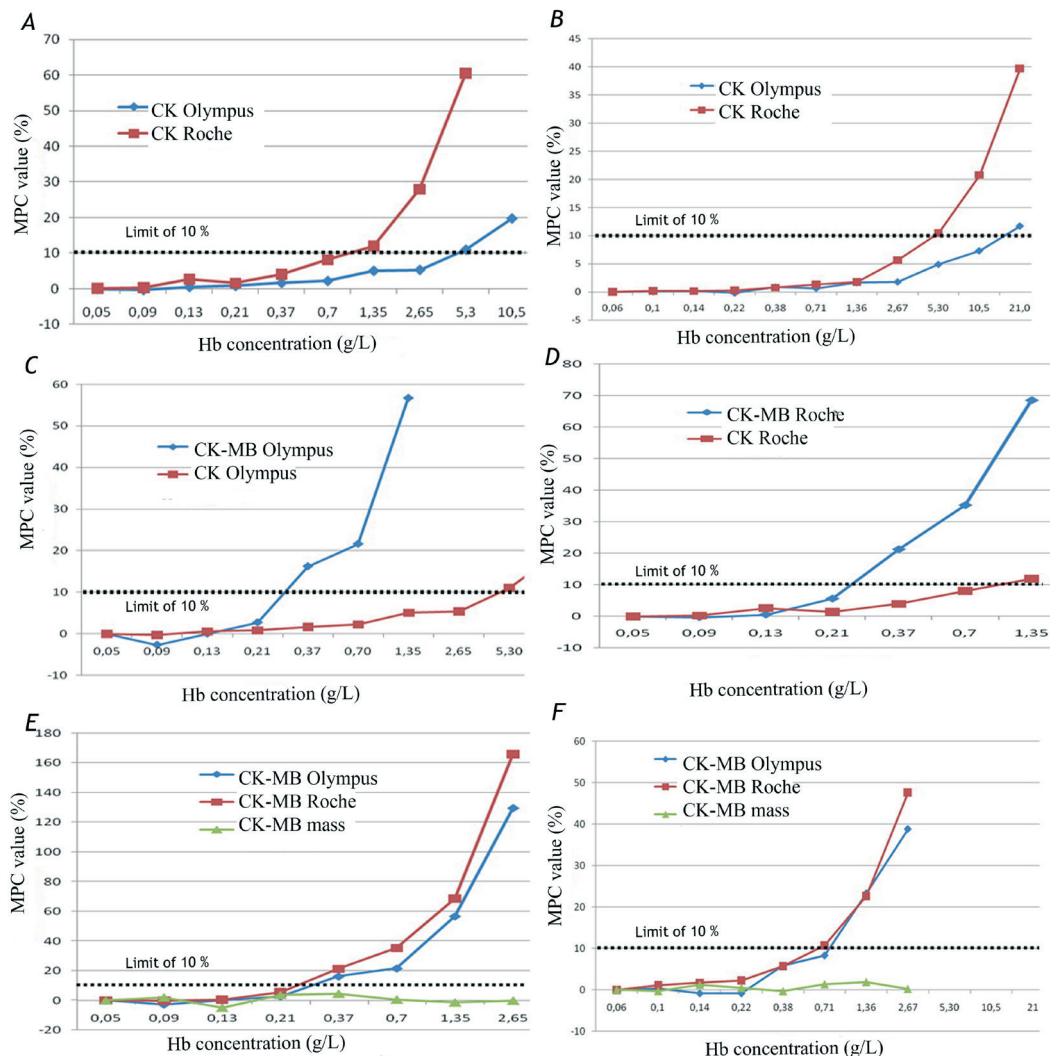
Figure 2. Interferographs; Mean Percent Change (MPC, %) of measured analytes and hemoglobin levels (g/L) in normal and pathologic serum pools. (MPC = [(C - C_0) /C_0] x 100. C, analyte concentration in sample with hemolysate; C_0, analyte concentration in sample without hemolysate)

A; MPC values for CK activity for Olympus and Roche in normal serum pool,
B; MPC values for CK activity for Olympus and Roche in pathologic serum pool.
C; MPC values for CK and CK-MB activity for Olympus in normal serum pool.
D; MPC values for CK and CK-MB activity for Roche in normal serum pool.
E; MPC values for CK-MB (mass) and CK-MB activity (Olympus and Roche) in normal serum pool.
F; MPC values for CK-MB (mass) and CK-MB activity (Olympus and Roche) in pathologic serum pool.

her biological variation values (Table 4). Therefore, this delayed effect can be considered reasonable. Using limit of RCV to evaluate interference can be problematic because analytical variation between laboratories is variable. In a study Ricos C et al. have determined RCV for 261 analytes including CK-MB and prepared a guide. They used 0.5 CV_I (desirable variation) instead of CV_A for analytes to achieve standardized RCV for clinical laboratories [22]. We can speculate that RCV for CK-MB activity in this guide can be used as limit for evaluating interference effect.

It has been previously demonstrated that hemolysis has less effect on mass assays than activity based methods but the degree and the direction of this interference is still contradictory in the literature [23]. Many studies

has claimed that hemolysis has not any effect on mass assays except at very high Hb values [24-26]. In contrast, Donnely detected positive interference due to hemolysis in CK-MB mass assays [27]. In another study, Kwon et al. [28] have shown that the samples with CK-MB level within reference range (CK-MB mass: <0.6 µg/L) were affected negatively by moderate (Hb: 5 g/L) to severe hemolysis (Hb: 10 g/L). In samples with CK-MB level higher than reference range (CK-MB mass: 9.4 µg/L) this effect was began at mild hemolysis level (Hb: >2.5 g/L). They determined the negative bias only in samples with troponin levels higher than reference range (Troponin: 6.3 µg/L) and no interference for myoglobin [28]. In the present study, it was not observed any interference up to the Hb level of 21 g/L for all three analytes for both
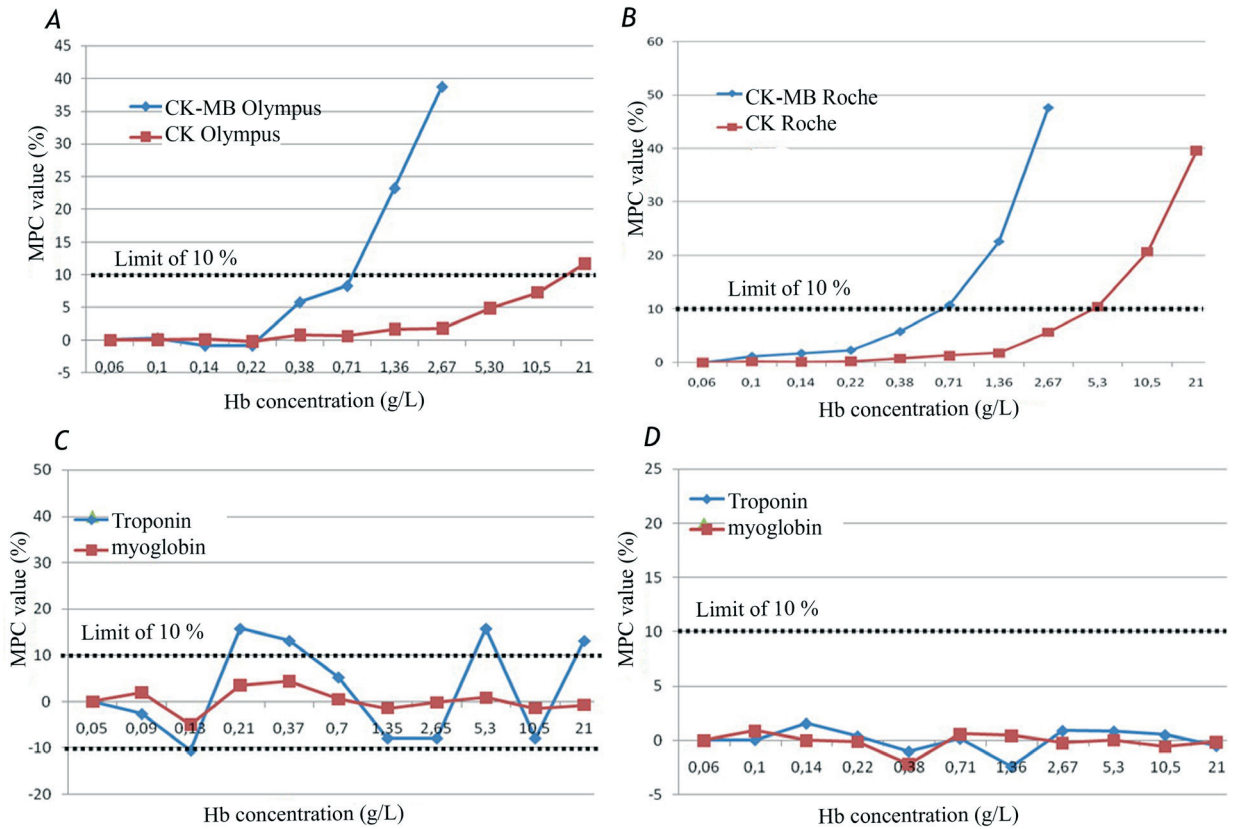
Figure 3. Interferographs; Mean Percent Change (MPC, %) of measured analytes and hemoglobin levels (g/L) in normal and pathologic serum pools. (MPC = [(C - C_0) /C_0] x 100. C, analyte concentration in sample with hemolysate; C_0, analyte concentration in sample without hemolysate)

A; MPC values for CK and CK-MB activity Olympus in pathologic serum pool.
B; MPC values for CK and CK-MB activity Roche in pathologic serum pool
C; MPC values for troponin and myoglobin in normal serum pool.
D; MPC values for troponin and myoglobin in pathologic serum pool.

Table 4. RCV values calculated for all analytes in normal and pathologic serum pools.

Analytes	z	CV_I	Normal serum pool		Pathologic serum pool	
			CV_A	RCV	CV_A	RCV
CK activity, U/L						
Olympus	1.96	22.8	5.21	64.8	3.46	63.9
Roche	1.96	22.8	2.44	62.9	1.50	62.7
CK-MB activity, U/L						
Olympus	1.96	19.7	7.77	58.1	1.77	54.3
Roche	1.96	19.7	6.74	57.1	1.66	54.2
CK-MB mass, µg/L	1.96	18.4	6.75	53.8	3.22	51.3
Troponin, µg/L	1.96	14	32.2	96.4	5.77	41.6
Myoglobin, µg/L	1.96	13.9	3.91	39.6	7.35	43.1

z score: 1.96 (for probability of 95%)
CV_I, intraindividual biological variation
CV_A, total analytical variation
RCV, Referance Change Value (RDD = $\sqrt{2}$ x z x $\sqrt{(CV_A^2 + CV_I^2)}$

Table 5. Calculated calibrator concentrations based on application parameters and data obtained from Olympus AU640 for CK and CK-MB (Olympus and Roche)

Analytes	ΔAbs/min	L/mol.cm	Total Volume μL	Sample Volume μL	F	C calculated U/L	C Calibrator U/L
CK-MB Olympus	0.0107	6300	156	6	4127	100	140
CK-MB Roche	0.0144	6300	312	12	4127	119	127
CK Olympus	0.0175	6300	153	3	8095	142	155
CK Roche	0.0419	6300	300	7	6803	285	304

ΔAbs/min, delta absorbance change value per minute, obtained from enzyme activity assay on the Olympus AU 640 analyzer.

F, IU/L (μmol/min/L) Factor used in the calculations of enzyme activity.
C calculated, calibrator value calcuated by molar absorbtivity (C = (ΔAbs / ε) x (Total volume/ sample volume)x 1/t(min) x 1/ light path (cm) x 10^6)
C Calibrator, calibrator value determined by manufacturer.

serum pools (Figure 2-C, D and Figure 3-C, D). Only MPC values for troponin in normal serum pool were close to the limit of 10%. But CV% values for troponin were also higher in normal serum pool (Table 1). But these limits were accepted as analytical variation, not the effect of interference. These results were also consisted with literature and kit inserts ensured by manufacturer [29,30]. Therefore we can say that performance of current analytical mass assays have gradually increased.

In conclusion, this study demonstrated that hemolysis effect on CK and CK-MB activity is began at lower Hb levels for Roche kits than Olympus and CK-MB activity is affected at lower hemolysis level than CK activity. Of course, performance of Roche kits might be different in Roche analytic systems. However, molar absorbtivity calculations suggests that there are still discrepancies at the standardization of CK-MB calibrators. And the different hemolysis effect on these commercial kits can be explained by this discrepancies. Mass assays for CK-MB are more reliable than activity based methods and should be preferred not only for hemolyzed, but all samples. Troponin and myoglobin assays are not affected by hemolysis.

Conflict of Interest: none

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