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DETERMINING LEUKOCYTE ALKALINE PHOSPHATASE SCORE WITH CYTOCHEMICAL STAINING

ODREĐIVANJE INDEKSA ALKALNE FOSFATAZE LEUKOCITA PRIMENOM CITOHEMIJSKOG BOJENJA

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Abstract
Introduction. Leukocyte alkaline phosphatase is an enzyme found in white blood cells, with its activity measured by the leukocyte alkaline phosphatase score. The aim of this study was to determine the optimal performing time and the primary biological sample in order to optimize the above laboratory analysis. **Material and Methods.** A prospective study was conducted from December 2023 to July 2024 at the Center of Laboratory Medicine, University Clinical Center of Vojvodina, involving 80 patients. Among them, 50 had confirmed leukocytosis with absolute neutrophilia, while 30 had normal white blood cell count. Leukocyte alkaline phosphatase was measured from capillary and venous blood samples immediately after collection, three hours post-collection, and seven days post-collection. All samples were prepared using the modified Kaplow method. The leukocyte alkaline phosphatase score is graded from 0 to 4 based on the ability of granulocytes to absorb certain amount of reagent according to the degree of enzyme activity. **Results.** The analysis revealed statistically higher leukocyte alkaline phosphatase scores in capillary blood samples compared to venous blood (62 (2-305) vs. 52 (5-292) vs. 50 (6-275) vs. 42 (2-280), p= 0.000). A significant decline of the score was observed in venous blood samples if the analysis is performed immediately after venipuncture, at 3 hours (52 (5-292) vs. 50 (6-275), p=0.021), and at 7 days post-sampling (52 (5-292) vs. 42 (2-280), p=0.000). **Conclusion.** Both the type of blood sample and the kinetics of the determination time affect the reliability of leukocyte alkaline phosphatase score results.
Key words: Alkaline Phosphatase; Leukocytes; Histocytochemistry; Blood Specimen Collection; Capillaries; Veins

Sažetak
Uvod. Alkalna fosfataza leukocita je enzim zrelih formi ćelija bele krvne loze čija se aktivnost meri indeksom alkalne fosfataze. Cilj ovog rada bio je da se utvrdi optimalno vreme izvođenja i primarni biološki uzorak u svrhu optimizacije prethodno navedene laboratorijske analize. **Materijal i metode.** Od decembra 2023. do jula 2024. godine u Centru za laboratorijsku medicinu Univerzitetskog kliničkog centra Vojvodine sprovedena je prospektivna studija koja je obuhvatila ukupno 80 pacijenata, od toga 50 ispitanika sa potvrđenom leukocitozom i apsolutnom neutrofilijom i 30 ispitanika sa fiziološkim vrednostima parametara kompletne krvne slike. Svim ispitanicima je određena vrednost indeksa alkalne fosfataze leukocita iz uzoraka kapilarne kao i venske krvi pripremljene neposredno, zatim nakon tri sata i sedam dana od momenta venepunkcije. Svi uzorci krvi su pripremljeni korišćenjem modifikovane *Kaplov* metode. Određivanje skora alkalne fosfataze leukocita zasniva se na sposobnosti granulocita da apsorbuju određenu količinu reagensa prema stepenu aktivnosti enzima i skorira se 0–4. **Rezultati.** Statistički više vrednosti indeksa alkalne fosfataze leukocita utvrđene su analizom iz uzoraka kapilarne krvi u odnosu na vrednosti dobijene iz venske krvi (62 (2–305) vs. 52 (5–292) vs. 50 (6–275) vs. 42 (2–280), p = 0,000). Postoji statistički značajan trend smanjenja vrednosti indeksa alkalne fosfataze leukocita dobijenih iz uzoraka venske krvi ako se analiza uradi odmah nakon venepunkcije i nakon tri sata (52 (5–292) vs. 50 (6–275), p = 0,021) kao i nakon sedam dana od trenutka uzimanja (52 (5–292) vs. 42 (2–280), p = 0,000). **Zaključak.** Vrsta uzorka krvi, kao i kinetika vremena određivanja imaju značaja na pouzdanost rezultata indeksa alkalne fosfataze leukocita.
Ključne reči: alkalna fosfataza; leukociti; histocitohemija; uzimanje uzoraka krvi; kapilari; vene

Introduction

Alkaline phosphatase (AP) is an enzyme that removes phosphate groups from molecules within different types of body cells [1]. As a basic biochemical test, elevated AP activity is detected in a range of pathophysiological conditions, including liver, kidney, and bone tissue disorders, as well as during physio-

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Abbreviations

AP	– alkaline phosphatase
LAP	– leukocyte alkaline phosphatase
CML	– chronic myeloid leukemia
UCCV	– University Clinical Center of Vojvodina
EDTA	– ethylendiaminetetraacid
CB	– capillary blood
VB0	– venous blood, zero hour
VB3	– venous blood, third hour
VB7	– venous blood, seventh day
CBC	– complete blood count
CG	– control group

logical growth and development [1, 2]. When detected in white blood cells, alkaline phosphatase is referred to as leukocyte alkaline phosphatase (LAP) [3]. While serum alkaline phosphatase measures total enzyme activity in the bloodstream, the LAP score specifically detects enzyme activity within white blood cells.

White blood cells are classified in two main lineages: myeloid and lymphoid, which are further divided into five major subtypes: neutrophils, eosinophils, basophils, monocytes, and lymphocytes. Neutrophils, a subtype of leukocytes containing AP, are the most abundant circulating leukocytes, comprising 50-70% of the total number of white blood cells. Neutrophil granulocytes play a crucial role in the innate immune system [4]. The alkaline phosphatase activity in neutrophil granules reflects LAP score fluctuations, providing insight into the state and degree of maturity of the observed cells [4–7]. As such, LAP activity varies significantly across different physiological and pathological conditions and is not associated with variations in serum alkaline phosphatase [5].

Although measurement of LAP scores is a highly subjective method, it remains a good starting point for the diagnosis and monitoring of chronic myeloid leukemia (CML) [8]. Moreover, LAP scores can be indicative in other conditions affecting the bone marrow or immune system disorders, such as infections, phases of blast crisis, or CML remission [9, 10]. Low LAP values are not due to altered structure or accelerated maturation of specific myeloid cell lineage but result from decreased enzyme content within leukocyte granules. Previous studies have ruled out catalytically defective enzyme synthesis as a cause of low LAP levels, confirming instead that these low values stem from insufficient synthesis of the functional enzyme [3, 5, 11].

The determination of LAP scores is based on the fact that leukocytes absorb certain amount of reagent responsible for specific staining inside the leukocyte cytoplasm. Microscopic examination then allows for subjective classification of staining intensity into five groups, scored from 0 to 4. If the sum of observed granulocytes falls within the range between 20 and 80, the result is considered to be within the reference range.

Despite its subjectivity, the LAP score remains a routine laboratory hematological analysis due to its quick turnaround time and low cost. Depending on the initial diagnosis, LAP score values can vary widely, from very low to very high. The aim of this study was to determine the optimal timing and type of primary biological sample for LAP score determination in order to optimize and standardize this laboratory analysis.

Material and Methods

A prospective study was conducted from December 2023 to July 2024. Blood samples from 80 patients were analyzed at the laboratory of the University Clinical Center of Vojvodina (UCCV) to determine their LAP values. We included 50 patients with confirmed leukocytosis with absolute neutrophilia in the complete blood count. Additional 30 participants with normal complete blood count were included as a control group. Capillary and venous blood samples were collected from all participants in the same laboratory in a single procedure. Venous blood was drawn from the cubital vein into EDTA tubes, while capillary blood was obtained from the palmar side of the distal phalanx of the third or fourth finger. For each participant, a drop of capillary blood was immediately applied to a glass slide to determine the LAP score. A second blood smear was prepared three hours after venous blood collection, as this timeframe was arbitrarily defined to represent the average delay between sample collection and the initial processing in the laboratory. At the same time, the second peripheral blood smear was prepared, fixed, and stored for seven days, after which it was stained, observed, and compared with previously obtained LAP results.

Blood samples were prepared using a modified Kaplow method [12]. After drying at room temperature, the preparations were fixed within 60 minutes of collection using a combination of methanol and formalin in a 10:1 ration, with the fixing process lasting for about 1 minute. The fixed slides were then rinsed with distilled water and dried at room temperature.

Next, the slides were placed in a Petri dish with a freshly prepared substrate solution consisting of 70 ml of Tris-hydroxymethyl-aminomethane, 60 mg of naphthyl phosphate, and 90 mg of Fast Red salt (pH= 9.2-9.4) for 30 minutes. was After incubation, the slides were rinsed under running water, dried at room temperature, and covered with Mayer hematoxylin solution for 15 minutes. Following the washing and drying steps, the preparations were observed and analyzed on the same day under a Nikon Eclipse E

400 electron microscope by two trained laboratory technicians.

Under the microscope, active granulocytes displaying a certain degree of discoloration were observed. A total of 100 neutrophil granulocytes were assessed, and their sum determined the final LAP score, which ranged from 0 to 400 depending on the number of stained leukocytes. Scores from 20 to 80 were considered within the reference range. LAP score was determined by the method of classifying leukocytes according to their enzyme activity. Activated neutrophil granulocytes release the enzyme alkaline phosphatase into the cytoplasm. LAP score determination was based on the ability of granulocytes to absorb the reagent, reflecting enzyme activity. This allowed subjective classification into five scoring groups. Absence of alkaline phosphatase activity in neutrophils was scored 0, while significant cytoplasmic staining due to a large amount of free enzyme and bound reagents received a score of 4. Scores 1, 2, and 3 were assigned based on shades in the discoloration of the cytoplasm of neutrophils. The normal distribution of continuous variables was assessed using the Shapiro-Wilk test. Data were presented as mean ± standard deviation for normally distributed continuous variables and median (min-max) for non-parametric continuous variables. Parametric (t-test) and non-parametric (Mann-Whitney) statistical tests were used. The obtained results were analyzed using the Data Analysis tool in Excel (Microsoft Corp, Redmond, WA). All data are presented in tables and graphs, accompanied by detailed legends for each analyzed parameter.

Results

The study included 34 male and 46 female patients. The average age of the female participants was 42 years, ranging from 19 to 73 years, while the average age of the male participants was 46 years old, with an age range of 18 to 74 years.

Analysis of complete blood count parameters revealed a statistically significant difference in the absolute number of leukocytes between the tested and control groups (14.48 (10.15-36.71) vs. 6.33 (4.20-8.46); p=0.002) as well as number of neutrophils (9.72 (5.57-25.8) vs. 2.70 (1.26-6.37); p=0.002). No statistically significant differences were observed in other measured parameters between the two groups **(Table 1).**

Further analysis showed that the leukocytosis group exhibited higher LAP score values across all sample modalities, but without statistical significance **(Table 2).**

A statistically significant difference was identified when comparing LAP scores obtained from capillary blood samples analyzed immediately after collection with those from venous blood samples analyzed immediately (Capillary blood (CB): 62 (2-305) vs. venous blood, zero hour (VB0): 52 (5-292), p=0.01), after 3 hours (CB: 62 (2-305) vs. venous blood, third hour (VB3): 50 (6-275), p=0.000), and after 7 days (CB: 62 (2-305) vs. venous blood, seventh day (VB7): 42 (2-280), p= 0.000). The highest values were observed in capillary blood samples analyzed immediately after sampling **(Table 3).**

Additionally, a statistically significant downward trend in LAP score was noted in venous blood sam-

Table 1. Results of descriptive statistics of complete blood count parameters

CBC parameters	Tested group (50)	CG (30)	p
Erythrocytes (x 10^9/L)	5.04 ± 1.09	5.65 ± 0.79	0.61
Thrombocytes (x 10^9/L)	352.5 (44-863)	212 (178-485)	0.250
Leucocytes (x 10^9/L)	14.48 (10.15-36.71)	6.33 (4.20-8.46)	0.002
Neutrophils (x 10^9/L)	9.72 (5.57-25.80)	2.7 (1.26-6.37)	0.002
Neutrophils (%)	64.69 ± 6.41	44.2 ± 14.52	0.090
Monocytes (%)	7.1 (3.60-21.80)	8.6 (6.8-10.32)	0.640
Lymphocytes (%)	25.55 ± 9.64	40.3 ± 12.12	0.090

Legend: tested group – group of participants with leukocytosis; CG – control group of participants with normal complete blood count; p – statistical significance

Table 2. Values of LAP results in the tested group and the control group of subjects

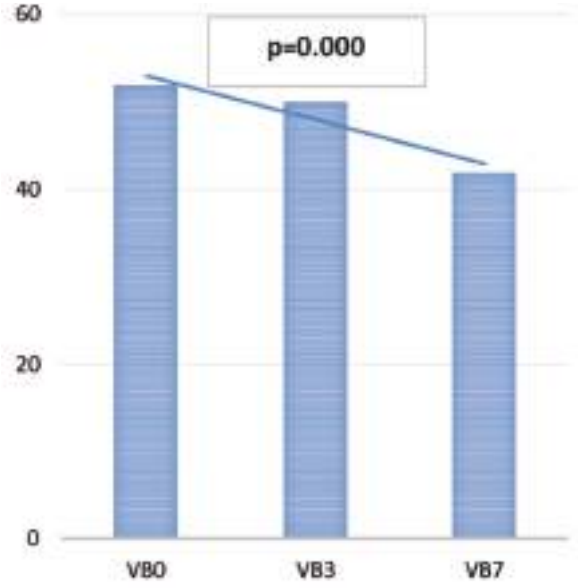
	$LAP_{tested\ group}$ (50)	LAP_{CG} (30)	p
CB	73 (2-305)	53 (30-129)	0.133
VB0	66 (5-292)	42 (26-111)	0.100
VB3	50.5 (2-280)	40 (15-108)	0.154
VB7	56.5 (6-275)	58 (22-95)	0.465

Legend: $LAP_{tested\ group}$ – leucocyte alkaline phosphatase index in the tested group LAP_{CG}–leucocyte alkaline phosphatase index in the healthy group; CB – capillary blood; VB0 – venous blood, zero hour; VB3 – venous blood, third hour; VB7 – venous blood, seventh day; p – statistical significance

Table 3. LAP score values in relation to the time of analysis and the type of sample from which the analysis was performed

CB (80)	VB0 (80)	VB3 (80)	VB7 (80)	p
62 (2-305)	52 (5-292)*	50 (6-275)**	42 (2-280)***	0.01* 0.000**, 0.000***

Legend: CB – capillary blood; VB0 – venous blood, zero hour; VB3 – venous blood, third hour; VB7 – venous blood, seventh day; *- p value between LAP values obtained from samples CB and VB0; ** - p value between the LAP values obtained from samples CB and VB3; *** - p value between the LAP values obtained from samples CB and VB7



Graph 1. Trend line of LAP score values obtained from venous blood samples in relation to the time of analysis
Legend: VB0 – venous blood, zero hour; VB3 – venous blood, third hour; VB7 – venous blood, seventh day; p – statistical significance

ples, with valued decreasing when analyzed immediately after venipuncture, after 3 hours (52 (5-292) vs. 50 (6-275), p=0.021), and 7 days after blood collection (52 (5-292) vs. 42 (2-280), p=0.000) **(Graph 1).**

Discussion

Leukocyte alkaline phosphatase activity plays an important role in differentiating leukemoid reaction from CML. This is achieved through cytochemical staining, which detects alkaline phosphatase activity in the neutrophils and bands [13]. Normal neutrophils and bands exhibit cytoplasmic alkaline phosphatase activity in the form of dark staining. Patients with leukemoid leukocytosis may exhibit very high LAP score. On the other hand, in patients with CML, the neutrophils show a very weak reaction to alkaline phosphatase staining, resulting in LAP score below the reference range. The simplicity and low cost of this laboratory test make it a useful tool for initial diagnosis of these conditions.

The reliability of this test is limited due to its subjectivity and experience of a technician performing the test. Also, the original method calls for completely fresh blood smears, but laboratory workflow constraints impose an alternative version, and routine analysis is performed from venous blood samples once a week. These facts were the starting point for re-examining the LAP score and its reliability in daily laboratory practice. The study included patients in whom varying LAP scores were expected. We compared LAP scores obtained from capillary and venous blood samples for all subjects, and found statistically higher LAP score values in capillary blood samples compared to venous blood samples, both immediately after collection and after seven days from the venous sampling **(Table 3).** Since this test is used to diagnose conditions where both slightly elevated and very low values are expected, standardized pre-analytical conditions is essential for accurate. Depending on the moment of processing, LAP score obtained from the venous blood samples continuously decreased **(Graph 1)**. As the method determined the reference range from 20 to 80, LAP score results can be interpreted incorrectly, and initially elevated values may be classified as normal, or referential ones pathologically lowered.

When analyzing LAP scores, peripheral blood samples are collected in vacutainers containing Ethylenediaminetetraacetic acid (EDTA), the most commonly used anticoagulant. However, literature suggests that EDTA may not be the optimal choice for alkaline phosphatase activity assays, even in routine biochemical testing [14]. Potassium EDTA, as an anticoagulant and chelating agent, interferes with calcium assays and clot formation, though it remains preferred for hematology testing. In most cases, relatively elevated EDTA levels due to insufficient sample volumes can intensify the chelation, which may interfere with reagent enzymes used for signal generation, such as alkaline phosphatase [15]. Based on this fact, we could assume that EDTA can also interfere with cytochemical staining, which may cause significant decline in the absolute values of the LAP scores in both patients with elevated and normal leukocyte counts.

Conclusion

Based on the study results, the highest leukocyte alkaline phosphatase score values were obtained from fresh capillary blood samples. At the same time, we proved that leukocyte alkaline phosphatase score results obtained from venous blood samples progressively decreased depending on the time of analysis - whether performed immediately or seven days after sampling.

However, the study has several basic limitations, including the small sample size, the subjectivity involved in microscopy and scoring, and the challenges in selecting non-hematological subjects. Future research would benefit from examining alternative modalities for determining and quantifying leukocyte alkaline phosphatase, such as flow cytometry or immunochemical chemiluminescence. By analyzing and validating these methods, one more key factor – the subjectivity of individuals performing the scoring during microscopic examination would be avoided.

References

1. Lowe D, Sanvictores T, Zubair M, John S. Alkaline phosphatase. Treasure Island: StatPearls Publishing; 2024.

2. Savić Lj, Savić D. Serum calcium and phosphorus concentration and alkaline phosphatase activity in healthy children during growth and development. Med Pregl. 2008;61(7-8):393-9.

3. Lipshitz J, Limaye S, Patel D. Leukocyte alkaline phosphatase score as a marker of severity and progression of myelodys-plastic syndrome. Blood. 2007;110(11):4625.

4. Tigner A, Ibrahim SA, Murray IV. Histology, white blood cell. Treasure Island: StatPearls Publishing; 2024.

5. Williams DM. Leucocyte alkaline phosphatase as a marker of cell maturity: a quantitative cytochemical and autoradiographic study. Br J Haematol. 1975;31(3):371-9.

6. Wiltshaw E, Moloney WC. Histochemical and biochemical studies on leukocyte alkaline phosphatase activity. Blood. 1955;10(11):1120-31.

7. Ullyot JL, Bainton DF. Azurophil and specific granules of blood neutrophils in chronic myelogenous leukemia: an ultrastructural and cytochemical analysis. Blood. 1975;45(4):469-82.

8. Kanegae MP, Ximenes VF, Falcão RP, Colturato VA, de Mattos ER, Brunetti IL, et al. Chemiluminescent determination of leukocyte alkaline phosphatase: an advantageous alternative to the cytochemical assay. J Clin Lab Anal. 2007;21(2):91-6.

9. Gantt S, Gervassi A, Jaspan H, Horton H. The role of myeloid-derived suppressor cells in immune ontogeny. Front Immunol. 2014;5:387.

10. Hughes A, Yong ASM. Immune effector recovery in chronic myeloid leukemia and treatment-free remission. Front Immu-nol. 2017;8:469.

11. Rosenblum D, Petzold SJ. Neutrophil alkaline phosphatase: comparison of enzymes from normal subjects and patients with polycythemia vera and chronic myelogenous leukemia. Blood. 1975;45(3):335-43.

12. Kaplow LS. Leukocyte alkaline phosphatase cytochemistry: apllications and methods. Ann N Y Acad Sci. 1968;155(2): 911-47.

13. Awale R, Maji R, Chandra D, Sharma S, Saxena R, Mukhopadhyay AK. Marked leucocytosis masquerading chronic leuke-mia: case series. IOSR Journal of Dental and Medical Sciences. 2016;15(11):65-9.

14. Bowen RA, Remaley AT. Interferences from blood collection tube components on clinical chemistry assays. Biochem Med (Zagreb). 2014;24(1):31-44.

15. Tate J, Ward G. Interferences in immunoassay. Clin Biochem Rev. 2004;25(2):105-20.

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