PREANALYTICAL PHASE IN HAEMATOLOGY
PREANALITIČKA FAZA U HEMATOLOGIJI

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Summary: The preanalytical phase is particularly important in haematology, where counts of particles and cells are performed in whole anticoagulated blood. The correct use and concentration of anticoagulant is mandatory to avoid spurious results, which can influence clinical decision. EDTA is the anticoagulant of choice, but it has some limits, especially for preserving stability and shape of platelets. Stability of haematological parameters is high, with the exception of leukocytes and reticulocytes. However, stability (and instrumental precision) should be evaluated together with biological variability and individuality index of various haematological parameters. Hematological tests are also influenced and interfered by high amounts of lipids and chylomicrons. The mixing procedure of the tubes after blood drawing and before analysis is also crucial for obtaining correct and valid data. There are some examples of interferences on automated haematological analysers which are used for diagnosing and screening pathological conditions. Cryoglobulins and erythrocytes parasites can induce spurious results of WBC, RBC and PLT, but the repeatability of these interferences could be used for alerting the pathologist and could reveal the presence of pathological proteins or blood parasites. New parameters have been proposed by modern haematological analysers, directly defined or calculated from traditional measures, but the clinical impact of these new parameters is often dependent on preanalytical variables.

Keywords: preanalytical phase, haematology, preanalytical variables

Kratak sadržaj: Preanalitička faza je naročito važna u hematologiji, gde se merenja čestica i ćelija vrše u celoj antikoagulisanoj krvi. Ispravna upotreba kao i koncentracija antikoagulansa je obavezna kako bi se izbegli nejasni rezultati, koji mogu uticati na kliničko odlučivanje. EDTA je preporučljivi antikoagulans, mada ima izvesna ograničenja, posebno u vezi sa očuvanjem stabilnosti i oblika pločica. Stabilnost hematoloških parametara je visoka, sa izuzetkom leukocita i retikulocita. Međutim, stabilnost (i instrumentalnu preciznost) treba ocenjivati zajedno sa biološkom varijabilnošću i indeksom individualnosti različitih hematoloških parametara. Na hematološke testove takođe utiču visoke koncentracije lipida i kilomikrona. Postupak mešanja epruveta posle uzimanja krvi a pre analize takođe je ključan za dobijanje tačnih i validnih podataka. Postoje primeri interferencije na automatizovanim hematološkim analizatorima koji se koriste za dijagnostikovanje i praćenje patoloških stanja. Krioglobulini i paraziti eritrocita mogu izazvati nejasne rezultate WBC, RBC i PLT, ali ponavljanje takvih interferencija može se iskoristiti za alarmiranje lekara i može ukazati na prisustvo patoloških proteina ili parazita u krvi. Moderni hematološki analizatori donose nove parametre, direktno definisane ili izračunate na osnovu tradicionalnih mera, ali klinički uticaj tih novih parametara često zavisi od preanalitičkih varijabli.

Ključne reči: preanalitička faza, hematologija, preanalitičke promenljive

Preanalytical factors are an important source of variation or errors in clinical laboratory measurements. The variation in specimen collection and effect of delay in sample pretreatment were examined in a specific study by collecting blood specimens from both arms of 10 persons from laboratory personnel at the Laboratory Centre of Tampere University Hospital. Samples from 14 adults including both healthy individuals and patients from the outpatient clinic were used to examine the effects of regional transportation. The published uncertainty estimates include the following components: biological variation (obtained from the literature), specimen collection from both arms, delay of pretreatment of specimens after collection, regional

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transportation (also used for in-house ambulatory patients attending hospitals) and analytical variation (uncertainty left after correction for known bias).

The measurement uncertainties of the erythrocyte count (RBC), mean corpuscular volume (MCV) and haemoglobin (Hb) concentration were small. The stability of the samples for haematological examinations was good except for platelets (PLT), which should be analysed without delay. The measurement uncertainty of leukocyte count (WBC) should ideally be estimated also at levels that are more significant in clinical patients (below 1×10^9/L and at 50×10^9/L), and in non-fasting conditions used for emergency patients. The transportation is widely increasing the measurement uncertainty of reticulocytes (Ret), which are characterized from high analytical variability, despite the introduction in routine laboratory of automated instruments (1). Therefore, the total variability of Ret is quite high and it could affect the clinical interpretation of data, especially when these results have a legal purpose (2).

There is a weak correlation between Hb and body mass index (BMI) in professional athletes belonging to different sport disciplines, whilst the correlation lacks for other haematological parameters. Therefore the variability of body mass is not deeply influencing the stability of haematological parameters (3).

The stability of haematological parameters is high: Hb and haematocrit (Ht) are stable for 48 hs, platelets and erythrocyte indexes for 24 hs. Leukocytes are stable for 48 hs, but the differential count must be done before 6 hs from blood drawing. The stability of Ret is lower than that of other haematological parameters and it is also dependent on analytical technique (4).

The Ret value measured on the Sysmex XT2000 in EDTA specimens stored at different temperatures during a interval period of 72 hours from the drawing decreased consistently after 4, 8, 24 and 48 hours at room temperature, but it appeared fairly stable in specimens stored at 2 °C. The main Ret values was stable up to a 72-hour storage period, but this was attributed to an artefact of the hematological systems. Although Ret, Ht and Hb values were substantially stable at 2 °C for 24 hours, the Ht exhibited a significant bias attributed to MCV modifications (5). The blood could be transferred at room temperature, but the 4 °C temperature is optimal, especially for platelets (6).

The blood drawing should be ideally performed when the subject is fasting. It is particularly important to obtain limpid serum and to avoid interferences due to turbidity on clinical chemistry analyses. Hematological tests are also influenced and interfered from high amounts of lipids and chylomicrons. Hyperlipemic samples could induce spurious results of Hb, usually increasing the values. For example, high values of MCHC (>360 g/L), due to a direct interference on spectrophotometric measurement of Hb, have been described in specimens from patients suffering from genetic or acquired hypertriglyceridemia (7) or from patients receiving intravenous emulsions (8). Very high concentrations of triglycerides (>6.59 mmol/L) interfere with haemoglobin measurement, as described on the Coulter instruments. When lipids form droplets having high volume, they could interfere the PLT count: an interference on platelet count on Bayer instruments was reported when triglyceride concentration was >3.44 mmol/L (8).

An interference on WBC and on specific scatterplots from subcutaneous adipose tissue has been reported in a sample obtained by traumatic femoral vein puncture (9).

High concentrations of glucose can induce spurious high values of MCV. During the dilution phase of blood, immediately after the aspiration of a sample into the instrument, RBC swell, because water is evoked by high quantities of glucose present into the RBC.

Very high blood concentrations of glucose (>27.75 mmol/L) induce osmolar effects on erythrocytes: MCV, MCHC and Ht are not accurate. Bayer technology seems to be especially sensitive to this effect, because RBC are isovolumetrically sphericized in these instruments (10).

There are some aspects of the preanalytical procedures which are sometimes neglected, but they affect analytical results, although with different levels of significance, because the alterations are not always clinically important. For example, the tourniquet application during a time of 2.30 ± 0.12 minutes on 27 professional cyclists induced the following modifications: Ht + 2.4 %, Hb +1.4 %, Ret −1.9%. It should be outlined that in four out of twenty-seven athletes the Ht modification was higher than analytical goals and it could be crucial for legal purposes (11).

The mixing procedure of the tubes after the blood drawing and before analysis is also crucial for obtaining correct and valid data. For example, when compared with the reference specimens inverted 6 times, results on unmixed specimens revealed significant decreases for red blood cell count, haemoglobin, haematocrit and platelets count, whereas the mean platelet volume was significantly increased (12).

The proper preparation and treatment of the tube is fundamental for obtaining correct data: overfilling the collection tube leads to inadequate sample mixing and setting of cellular contents: all the parameters are altered (13).

Circadian rhythms are faible for haematological parameters.

The nadir (lowest value during the 24 hs) for Ht is showed during the night with a variation into the day of 5% (from 45.3 ± 3.1 to 42.9 ± 1.5%, on 8 individuals); the variation was higher when submaximal exercise was held. RBC, Hb, and Ht show low amplitude

circadian rhythm, with an acrophase (highest part of the normal fluctuation) at 11:00, which is, however, lower than analytical variability. WBC have the acrophase at evening (21.00–24:00) with a variation of 0.9–2.0 x 10^9/L. Ret show the acrophase at 01:00 (between 20:00 and 04:30; the fluctuation is higher than analytical variability; the standardization of the drawing at early morning is necessary.

A circaseptan rhythm for RBC, Hb and Ht was reported, with the acrophase at Monday. Seasonal variations determine haemodilution on summer (increase of 9% of plasmatic volume) (14).

EDTA is the anticoagulant of choice for hematological testing (15).

The EDTA pH varies on the basis of the salt type: the EDTA acidity decreases when the number of ions increases. The free acid solution of EDTA shows a pH of 2.5±1.0, whilst the K_3 salt (1% solution) is characterized by a pH of 7.5±1.0. The EDTA salts are hyperosmolar, causing water loss from cells. The cell shrinkage is less apparent when K_2 and Na_2 EDTA are used. Microhaematocrit, the reference International Council for Standardization in Haematology (ICSH) method for packed cell volume (PCV), is not influenced by K_2 and Na_2EDTA, whilst it is decreased by K_3EDTA. Thus, K_2 should be preferred to K_3 salt, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (16, 17).

Nevertheless, automatic hemocytometers measure the mean corpuscolar volume (MCV) and calculate PCV. The effect of cell shrinkage should not be clinically important. On the contrary, the concentration is crucial. Dacie & Lewis proposed a K_2EDTA concentration of 1.5±0.25 g/L and a similar concentration is recommended by NCCLS (1.5–2.2 g/L), whereas ICSH recommends a concentration of 4.55 mmol/L of blood (18). When the concentration of EDTA is increased, the MCV measured by automatic instruments is variably influenced, but it basically tends to rise. This effect is less evident when K_3 is used (19).

Hirudin, a polypeptide of 7 KDa produced from salivary glands of leeches, inhibits coagulation at very low doses (10 μg per 1 mL of blood) and it does not require the presence of antithrombin. Hirudin irreversibly binds by the carboxyterminal tail to the fibrinogen binding site of thrombin, inhibiting the conversion of fibrinogen to fibrin. In a recent investigation, results of laboratory testing in samples collected in 4 mL tubes containing a final hirudin concentration of 1000 antithrombotic activity units per ml were compared to those obtained in samples collected by traditional K2 EDTA and serum tubes (20). Overall, results of CBC and hematological parameters were rather satisfactory. A lower correlation was only recorded for monocytes and basophils; monocytes were occasionally recognized as basophils on the Coulter STKS.

EDTA is not able to stabilize completely the PLTs,

allowing some morphological alterations to occur. When in contact with EDTA, PLTs undergo a time-dependent modification from a discoidal to a spherical shape. Although several modern hemocytometers measure the mean PLT volume (MPV), its clinical use has been limited by some preanalytical pitfalls. MPV may be a valid clinical finding for detecting the source of a thrombocytopenia. MPV is normal in the autoimmune thrombocytopenia, while it is increased in disseminated intravascular coagulation (DIC), microangiopathies or in pathologies impairing PLTs maturation and release (21). There is a consistent inverse association between number and volume of PLTs, as demonstrated by Bessman (22); such a relationship is linear up to 400 x 10^9/L PLT. The modification of the MPV depends on the time of contact with the anticoagulant. This mechanism involves a modification of the membrane permeability, through a cyclic AMP (cAMP) mediated reaction. The PLT undergoes a spherical change, yielding to an apparent increase of the volume when the particle passes through an impedance-based analyzer. The volume increase is evident in the 60 minutes following the blood drawing and becomes further stable within three hours. Therefore, the MPV should be always quantified at fixed time from the blood drawing to allow reliable intra (longitudinal) and inter-individual comparisons (23).

The apparent increase of the PLT volume is generally observed by using impedance analyzers. In light-scattering-based hemocytometers, where hematological parameters are defined by both volume and light refraction indexes, the measured MPV is more variable. MPV is usually decreased in these systems, though a less frequent increase can be recorded in a third of the cases. When the two technologies were compared, there were no significant differences for PLT count, whilst MPV of light scattering-based hemocytometers was lower than of the MPV impedance-based ones (24).

Anticoagulants other than EDTA have been proposed to obtain correct and valid measurements of MPV, including ACD (adenosine, citrate and dextrose) and Na_2EDTA, sodium citrate and PGE1 (prostaglandin E1) (22), CTAD (citrate, theophylline, adenosine, dextrose) and pyridoxalphosphate, which have been validated on impedance-based systems (15).

The EDTA-induced pseudothrombocytopenia may be detectable on some automated systems by means of flags and graphics, and can be differentiated by comparing data obtained on EDTA- and sodium citrate-collected specimens. The pseudothrombocytopenia may be associated with negative outcomes if left undetected, triggering unnecessary further investigation, unjustified and invasive pharmacological or medical treatments (25). The EDTA-induced pseudothrombocytopenia can be observed in either health or disease and they are not related to gender and age. The association with dysfunctions or anomalies of PLT is rare (26). As reported in several epidemiological stud-

ies (27–32), the prevalence of EDTA-induced pseudothrombocytopenia is nearly 0.1% and its frequency appears higher in thrombocytopenic patients, ranging from 1.25% to 15.3% (33).

Apparently, this form of spurious thrombocytopenia is caused by IgM autoantibodies directed against the glycoproteins IIa and IIIb on the PLT surface. In fact, PLTs of patients with Glanzmann disease, which is characterized by the lack of expression of the IIa/IIIb complex, do not react with autoantibodies of the pseudothrombocytopenic subjects (34).

The IIa/IIIb complex is crucial for PLT adhesion, the first step of aggregation and thrombus formation. The EDTA may induce modifications of the structural morphology and externalization of the complex, triggering the immunological reaction with the autoantibodies.

The phenomenon does not appear to be mediated by calcium chelation and it has been reported also for molecules similar to the EDTA, as the ethylentriamminopentaacetic acid (35). Moreover, the kinetic is time- but not temperature-dependent (36); it suddenly appears within two hours from the blood drawing, as demonstrated by a case occurred during the hospitalisation of the patient (37).

There are some examples of interferences on automated haematological analysers which are used for diagnosing and screening pathological conditions. Cryoglobulins and erythrocyte parasites can induce spurious results of WBC, RBC and PLT, but the repeatability of these interferences could be used for alerting the pathologist and reveal the presence of pathological proteins or blood parasites. Cryoglobulins have been defined as serum proteins with temperature-dependent solubility, representing anomalous increases of the normal protein constituents of serum. The paraproteins associated with myeloma or immunoproliferative disorders are often cryoglobulins. The precipitation of cryoproteins depends on their concentration, the immunoglobulin class to which they belong, and the pH of the medium (38).

The idiopathic cryoglobulinaemia is characterized by a triad of symptoms: purpura, arthralgia and weakness, often accompanied by renal failure, but such a condition is often linked with many different diseases (secondary cryoglobulinaemia). A commonly used classification includes three types of cryogobulins (39): single monoclonal immunoglobulin, characteristically found in patients with multiple myeloma or Waldenstrom's macroglobulinaemia, usually IgM, less frequently IgG, and rarely IgA; monoclonal-polyclonal (mixed cryoglobulins); mixed polyclonal cryoglobulins, associated with infection or inflammatory disease or, less commonly, idiopathic. Traditionally, to screen for the presence of cryoglobulin, serum should be stored at 4 °C for several days and checked for the appearance of a precipitate which is completely reversible on rewarming. The precipitate could be quantified, after centrifugation of serum in a tube.

The presence of cryoglobulin may affect hematological laboratory tests. On automated analysers of the Coulter series cryoglobulins many years ago were described to produce pseudoleukocytosis and/or pseudothrombocytosis, because the globular or cylindric precipitates formed by proteins are counted as cells or particles (38, 40). With other automated counters, such as the Technicon ones, which use prewarmed reagents and a WBC diluent at low pH (3.2) cryoprecipitation does not occur (41): the cryoproteins do not precipitate at pH <5.0 or > 8.0. In Technicon instruments, however, the interference is evident on the PLT count, showing a hyperbolic curve of distribution very similar to the WBC one in impedance systems (42).

The occurrence of a cryoprecipitate in the Coulter instruments in cases of cryoglobulinaemia, rather than being a source of inaccurate results, represents an important finding in the laboratory detection of cryoglobulins. We described the possible use of pseudoleukocytosis and the correspondent typical hyperbolic shape of WBC curve for screen the presence of cryoglobulins (43).

The elevated sensitivity (10 flagged of 14 cases), the elevated specificity (flag present in some patients with cryocrit as low as 3% and lack of false-positive results), and the good reproducibility (all the cases followed were confirmed) allow us to consider the interference of cryoprecipitable proteins in the Coulter pattern (giving spurious, but easily recognizable results), an important tool in the detection of cryoglobulinaemia even in unsuspected patients. The WBC histogram observed in cryoglobulinaemias seems to be specific for this disease, as it has never been found in other conditions. In some cases, the cryoprecipitates are small and they are not able to affect the WBC count, but they interfere with the differential, while classified as lymphocytes (44).

The description of some spurious results induced by the presence of malaria parasites concerned single cases: in one patient treated for malaria, small RBC infected by trophozoites of Plasmodium falciparum were misinterpreted as PLT by the analyser, leading to a spuriously normal PLT count (45). The increase in sensitivity of analysers permitted the accurate report of fixed interference of malaria parasites on neutrophils lobularity plot in Abbott Cell Dyn, inducing a peculiar additional cloud represented from the parasited RBCs. A study using the Abbott Cell-Dyn 4000 and laser light depolarization analysis found that cases of treated convalescent malaria with no residual parasitemia demonstrated abnormal depolarization patterns. This is explained by the kinetics of hemozoin clearance. The removal of pigment-containing monocytes is slower (median, 216 hours) than parasitized erythrocytes (median, 72 hours) (46). A study screened the parasites through the Coulter VCS analysis method. This detection method depends on changes in the SD vo-

lumes of lymphocytes and monocytes in the presence of malaria. By using a calculation, a »malaria factor« is produced. A malaria factor greater than 3.7 was an indicator of malaria infection: the specificity of the method was 94% and the sensitivity 98%. False-positive results from this study were almost exclusively from samples from patients with infections other than malaria and patients with HIV. Some bacterial and viral infections including HIV seem to increase the standard devi-

ation (SD) of the volume of lymphocytes and therefore may cause false-positive results for malaria when the malaria factor is used for diagnosis. When a malaria factor of greater than 3.7 is used in conjunction with the algorithm using the SD volume of the monocytes, mean volume of monocytes, low platelet and eosinophil counts, and the presence of a peak in the WBC threshold monitor histogram to detect malaria, specificity is improved (47).

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