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Effect of temperature variations on clinical biochemical assays

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Abstract

Incubation temperature is a critical factor in measuring biochemical assays, therefore this study was designed to measure effect of temperature variations on the some important clinical assays which are C - reactive protein (CRP), vitamin D, D-Dimer, serum ferritin and rheumatoid factor (RF). Blood samples were collected from fifty different patients and only samples closer to the normal value were used in this study. All tests were incubated at room temperature (RT) and 37 °C in five replicates. Results showed that there was a significant effect of temperature variations on these biochemical assays. It could be concluded that incubation at 37 °C is the best temperature to perform chemical assays.

Keywords: C-reactive protein, Biochemical assays, temperature variation, vitamin D, D-Dimer.

Introduction

Biochemical laboratory tests are fundamental tools in clinical diagnostics, providing critical information about organ function, metabolic processes, enzyme activity, and the homeostasis of electrolytes and other biomolecules[1]. Elevated temperatures can accelerate enzymatic activities and biochemical reactions, leading to the degradation of temperature-sensitive analytes such as glucose, enzymes, and certain proteins. For instance, glucose levels may decrease rapidly due to ongoing glycolysis by metabolically active cells if samples are not properly cooled[2]. Conversely, low temperatures may inhibit enzymatic reactions, alter analyte solubility, or induce protein and salt precipitation potentially leading to inaccurate measurements[3]. Moreover, temperature variations during sample collection, transportation, and storage can affect the integrity of plasma, serum, and other biological fluids. This may result in hemolysis, protein denaturation, or changes in the concentration of analytes, thus compromising the validity of laboratory data[4]. The liver synthesizes CRP in response to pro-inflammatory cytokines, particularly interleukin-6 (IL-6), which is released during inflammation. CRP's primary function involves binding to dead or dying cells and certain pathogens, activating the complement system and facilitating phagocytosis. This process helps the immune system clear harmful agents from the body. CRP levels in the blood can rise dramatically in response to an inflammatory stimulus, making it a useful marker for detecting inflammation and monitoring its resolution[5]. The vitamin D test measures the concentration of 25(OH) D in serum or plasma to evaluate vitamin D sufficiency, insufficiency, or deficiency. This test is crucial for diagnosing

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conditions such as rickets, osteomalacia, osteoporosis, and other disorders related to calcium metabolism. Additionally, low vitamin D levels have been linked to an increased risk of chronic diseases, including cardiovascular diseases, diabetes, certain cancers, and immune-related conditions[6,7]. Serum 25(OH)D is commonly measured using techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and immunoassays, with LC-MS/MS considered the gold standard due to its high specificity and accuracy[8]. Rheumatoid Factor (RF) is an autoantibody, typically of the IgM class, that targets the Fc region of immunoglobulin G (IgG). The presence of RF in the blood is a key biomarker used in the diagnosis and management of autoimmune diseases, particularly rheumatoid arthritis (RA). Although RF is not specific to RA, it is detected in approximately 70–80% of patients with the disease, making it an important tool in clinical biochemistry for assessing autoimmune activity[9].

The RF test is commonly performed using immunoassays such as nephelometry, turbidimetry, or enzyme-linked immunosorbent assays (ELISA), which quantify RF levels in the serum. Elevated RF levels are associated with more severe disease progression, joint damage, and extra-articular manifestations in RA. However, RF can also be detected in other conditions, including systemic lupus erythematosus (SLE), Sjogren's syndrome, chronic infections, and even in healthy individuals, especially the elderly[10]. Due to its limited specificity, the RF test is often combined with other markers, such as anti-cyclic citrullinated peptide (anti-CCP) antibodies, which improve diagnostic accuracy for RA. Despite its limitations, RF remains a valuable marker for early detection, prognosis assessment, and monitoring therapeutic responses in autoimmune diseases[11]. The measurement of D-dimer is typically performed using quantitative immunoassays, such as enzyme-linked immunosorbent assays (ELISA), latex agglutination tests, and immunoturbidimetric assays. High-sensitivity D-dimer assays are preferred in clinical practice due to their ability to detect low concentrations, improving diagnostic accuracy, especially in low-risk patients[12]. Ferritin is a vital intracellular protein complex responsible for storing and regulating the release of iron in a controlled manner.

It plays a crucial role in maintaining iron homeostasis, protecting cells from the toxic effects of free iron, and ensuring its availability for essential biological processes such as oxygen transport, DNA synthesis, and cellular respiration[13]. In biochemistry, serum ferritin is widely used as a clinical biomarker to assess the body's iron stores. Although ferritin is primarily stored in the liver, spleen, and bone marrow, a small amount circulates in the bloodstream. Serum ferritin levels correlate with total body iron, making it a key diagnostic marker for conditions such as iron deficiency anemia, iron overload disorders (e.g., hemochromatosis), and chronic inflammatory diseases. Interestingly, ferritin also acts as an acute-phase reactant, with levels rising during inflammation, infection, and malignancy, independent of iron status[14]. The measurement of serum ferritin is typically performed using immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA) or chemiluminescent immunoassays, providing accurate quantification for clinical evaluation[15]. Based on what mentioned above, the current study was designed to compare between results of clinical biochemical assays incubated at room temperature (RT) and 37 °C.

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Materials and Methods

Ethics and Consent

All patients and healthy volunteers were asked for their permission to participate in this study. The research was ethically approved by the Committee of College of Science / Al-Nahrain University.

Sample Collection and processing

All participants were asked for their history of any chronic diseases or disorders. Blood samples were collected and processed according to standard protocol. For serum collection, 5 ml of vein whole blood in gel tubes was allowed to clot at room temperature for 30 minutes then centrifuged at 3500 rpm for 5 minutes at room temperature. Serum was used to measure concentrations of C- reactive protein (CRP), ferritin, vitamin D3 and rheumatoid factor (RF). For plasma collection, 2.5 ml of vein whole blood was collected in sodium citrate tubes and centrifuged at 1500 rpm for 15 minutes. AFIAS-10 machine and kits (BodiTech / South Korea) were used to process all tests.

Procedure

All cartridges and samples were brought to stand at room temperature (RT) 30 minutes prior to performing the assay. A volume of 200 µl of serum and plasma was added to assigned well of the sample on the cartridge. Each sample was analyzed in duplicate; one cartridge was kept at RT and the other cartridge was incubated at 37 °C for 15 minutes. For each assays, 5 samples (n=5) were assessed. After elapsing the incubation time, cartridges were analyzed through the AFIAS machine. Data were collected and results were analyzed and plotted using excel software. In case of healthy volunteers, results close to upper limit of the normal values were chosen while those close to the lower limits of normal values were excluded.

Statistical analysis

GraphPad Prism software version 6 was used to analyze data. Sample number (N=5) for each assay were applied. The data was ± standard deviation (SD) and variations between data were considered as significant when p value was 0.05 and less.

Results and Discussion

In this study the results of all assays revealed variations in values when incubated at RT compared to their results incubated at 37 °C. As shown in figure (1a), CRP results of 5 healthy samples appeared normal when incubated at RT while they were abnormal when incubated at 37 °C with mean variation from 7.9 mg/l to 13.9 mg/l respectively. On the other hand, unhealthy samples incubated at RT had lower values than those incubated at 37 °C with mean of 23.4 mg/l and 47.2 mg/l respectively as demonstrated in figure (1b). These variable mean values according to the interactions between thermal energy and the stabilizing and destabilizing forces acting on a protein molecule in solution and which are complex, but to a first approximation, increases in temperature will favor destabilization of protein structure and a sufficient input of heat energy will ultimately lead to protein denaturation[16].

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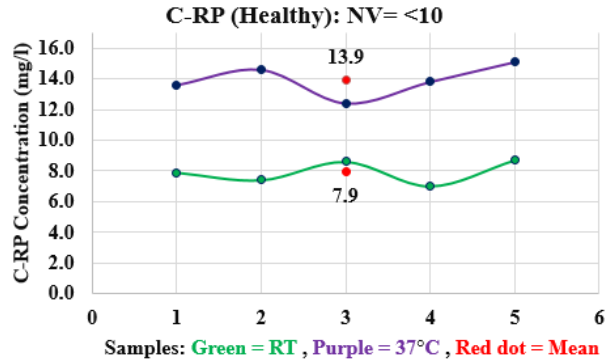
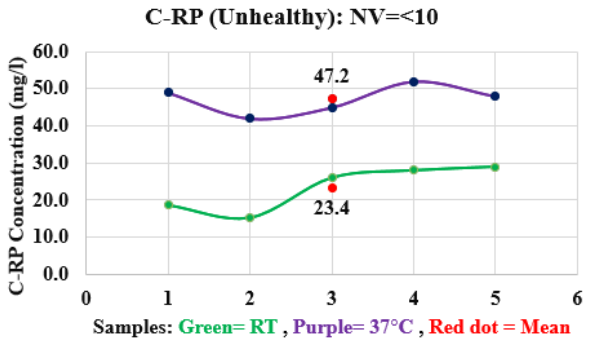


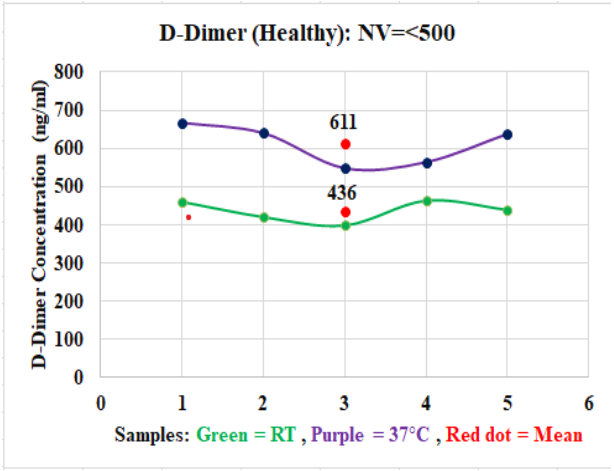
(a) CRP levels of healthy samples



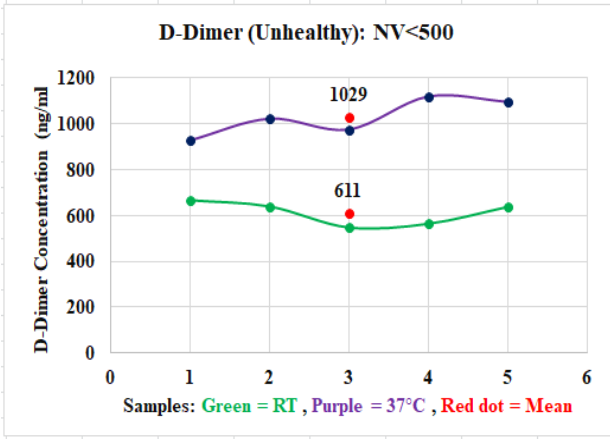
(b) CRP levels of unhealthy samples

Figure (1): Effect of temperature variation on levels of CRP. (a) Healthy sample, (b) unhealthy samples.

D-Dimer is an invaluable analyte in evaluating thrombin and plasmin activity and is specific for fibrin derivatives[17]. This study evaluated the effects of variation temperatures on D-dimer stability as shown in figure (2a), D-Dimer results of 5 healthy samples appeared normal when incubated at RT while they were abnormal when incubated at 37 °C with mean variation from 436 ng/ml to 611 ng/ml respectively. On the other hand, unhealthy samples incubated at RT had lower values than those incubated at 37 °C with mean of 611 ng/ml and 1029 ng/ml respectively as demonstrated in figure (2b), these changes in D-Dimer values could result from a combination of protein degradation because the effect of temperature changes[18].



(a) D-Dimer levels of healthy samples



(b) D-Dimer levels of unhealthy samples

Figure (2): Effect of temperature variation on levels of D-Dimer. (a) Healthy sample, (b) unhealthy samples.

Serum ferritin results of 5 healthy samples as shown in figure (3a) appeared normal when incubated at RT while they were abnormal when incubated at 37 °C with mean variation from 38.7 ng/ml to 61.7 ng/ml respectively. On the other hand, unhealthy samples incubated at RT had lower values than those incubated at 37 °C with mean of 20.8 ng/ml and 33.8 ng/ml respectively as demonstrated in figure (3b). From the above results found the best reaction result were in 37 °C where ferritin is a blood protein that

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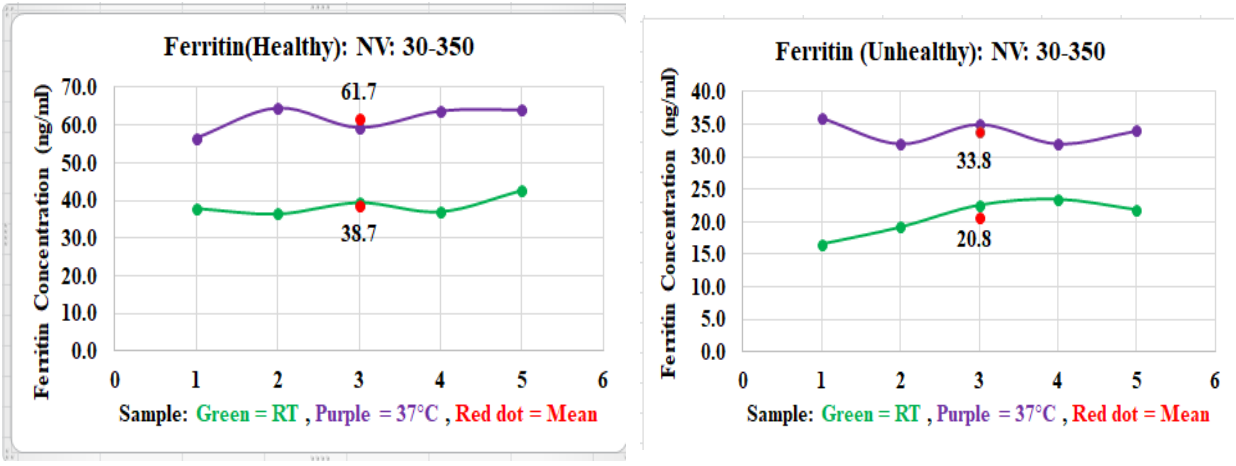
contains iron and affected by changes of temperature. Therefore protein stabilities are typically determined by gradually changing temperature or the concentration of a chemical denaturant and measuring the unfolding by a spectroscopic technique or calorimetry[19].



(a) Serum ferritin levels of healthy samples **(b) Serum ferritin levels of unhealthy samples**

Figure (3): Effect of temperature variation on levels of serum ferritin. (a) healthy sample, (b) unhealthy samples.

Serum 25(OH) D concentrations changed according to air temperature. As shown in figure (4a), vitamin D results of 5 healthy samples appeared normal when incubated at RT while they were abnormal when incubated at 37 °C with mean variation from 26.7 ng/ml to 40.5 ng/ml respectively. On the other hand, unhealthy samples incubated at RT had lower values than those incubated at 37 °C with mean of 6.7 ng/ml and 11.4 ng/ml respectively as demonstrated in figure (4b). There is a positive correlation between temperature and vitamin D levels, this is due to the specific enzymes involved in the metabolism of vitamin D, 25(OH) D and 1, 25(OH)2D play important roles in regulating the bioavailability of these molecules. For a reaction to occur between an enzyme and substrate the two molecules need to collide.

At lower temperatures the reaction rate is low as the enzymes and substrates do not collide frequently. As temperature increases, reaction rates generally rise due to more frequent collisions between enzyme and substrate molecules. Once the temperature exceeds an enzyme's optimal range, the enzyme can denature, losing its structure and function, which significantly decreases or stops the reaction. Human enzymes have an optimal activity at about 37 degrees Celsius[21].

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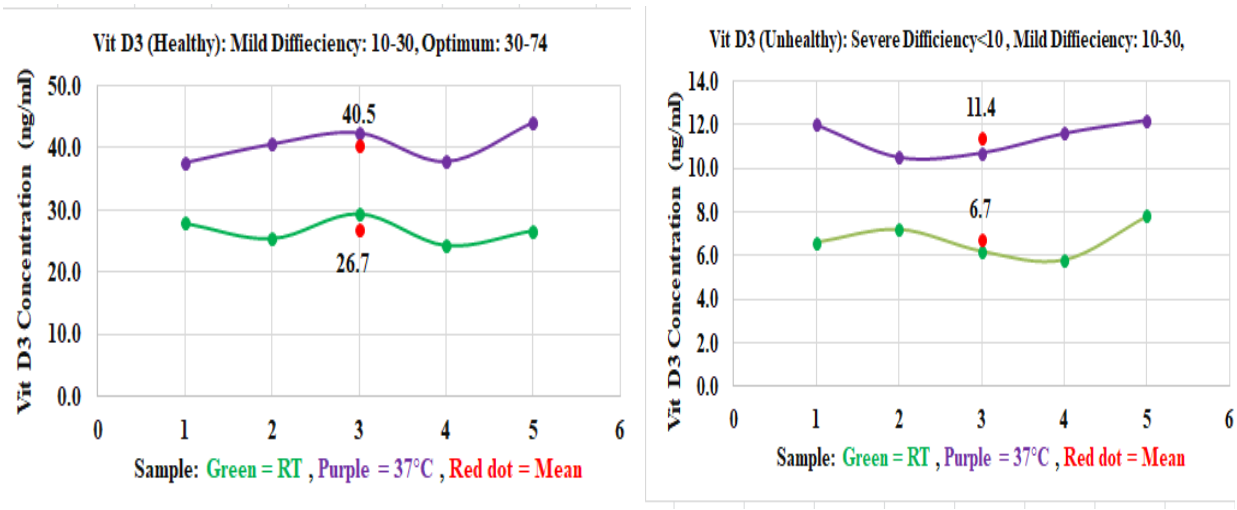

(a) Vitamin D levels of healthy samples **(b) Vitamin D levels of unhealthy samples**

Figure (4): Effect of temperature variation on levels of vitamin D. (a) Healthy sample, (b) unhealthy samples.

Rheumatoid factors are proteins made by the immune system. As shown in figure (5a), RF results of 5 healthy samples appeared normal when incubated at RT while they were abnormal when incubated at 37 °C with mean variation from 13.1 IU/mL to 16.7 IU/mL respectively. On the other hand, unhealthy samples incubated at RT had lower values than those incubated at 37 °C with mean of 18.1 IU/mL and 26.4 IU/mL respectively as demonstrated in figure (5b). This variable in results due to increases in temperature will favor destabilization of protein structure and a sufficient input of heat energy will ultimately lead to protein denaturation[16] and that is prefer the incubate the reaction test at 37 °C. A recent study by (Patel *et al*. 2024) examined the effect of temperature on the binding affinity of protein-ligand complexes and found that temperature-induced conformational changes could enhance or reduce binding affinity[22].

The temperature dependence of enzyme activity is another area where recent studies have provided valuable insights. Enzymes are proteins that catalyze biochemical reactions, and their activity is highly sensitive to temperature. Studies have shown that enzymes exhibit optimal activity at certain temperatures, with activity decreasing significantly at temperatures either lower or higher than the optimum. This phenomenon is critical in understanding metabolic processes in organisms and the application of enzymes in industrial processes. In contrast, mesophilic enzymes tend to lose their activity more quickly as temperature rises[23].

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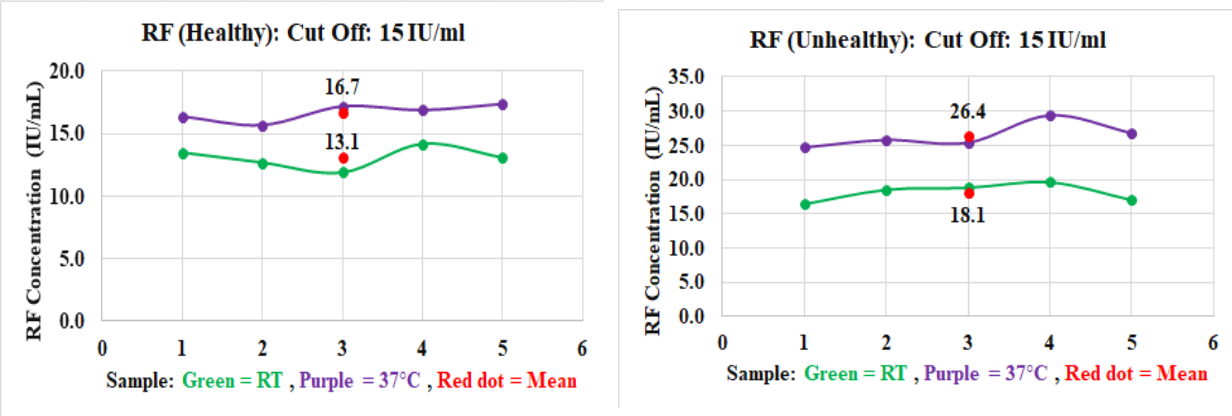

(a) RF levels of healthy samples **(b) RF levels of unhealthy samples**

Figure (5): Effect of temperature variation on levels of RF. (a) Healthy sample, (b) unhealthy samples.

More clarification for the results could be explained according to the rate of chemical reactions is significantly influenced by temperature variations. Generally, increasing the temperature accelerates the reaction rate, while decreasing it slows the reaction. This phenomenon can be explained through several key principles[24].

1. Collision Theory:

As temperature rises, molecules gain kinetic energy, leading to more frequent and energetic collisions between reactant molecules. For a reaction to occur, these collisions must have sufficient energy, known as the activation energy, to break existing bonds and form new ones. Elevated temperatures increase the number of molecules possessing the necessary activation energy, thereby enhancing the likelihood of effective collisions and speeding up the reaction rate.

2. Arrhenius Equation:

The Arrhenius equation mathematically describes the relationship between temperature and reaction rate:

$$k = A \times e^{-\frac{Ea}{RT}}$$

Where:
k is the rate constant,
A is the pre-exponential factor,
Ea is the activation energy,
R is the universal gas constant,
T is the absolute temperature in Kelvin.

This equation illustrates that even a modest increase in temperature can lead to a significant rise in the rate constant, thereby accelerating the reaction.

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3. Empirical Observations:

Empirical data supports these theoretical insights. For instance, it's commonly observed that for many reactions, a 10°C increase in temperature can double or even quadruple the reaction rate. This is because higher temperatures result in a greater proportion of molecules achieving the activation energy necessary for the reaction.

Recent studies delves into how elevated temperatures affect reactions on metal surfaces[25]. The research highlights that at high temperatures, factors such as the local chemical environment, surface atom mobility, and substrate thermal expansion play crucial roles in reaction dynamics. These findings deepen our understanding of temperature effects on reaction rates, especially in industrial applications involving metal catalysts.

Conclusions

Temperature plays a critical role in results of biochemical assays. Each assay has proper temperature that gives the most realistic result. This study has confirmed that chemical assays should be performed at 37 °C to obtain the most accurate results.

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Author's Declaration

No conflict of interest.

Author's Contribution Statement

The paper was fully achieved by one author (Husam E. Salman).

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