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# Determination of N-acetyl-β-hexosaminidase in serum from hemolyzed blood

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ABSTRACT

**Background:** Determination of lysosomal N-acetyl-β-hexosaminidase (HEX) in serum from hemolyzed blood, creates serious analytical problems, because hemoglobin absorbs light at a similar wavelength like 4-nitrophenol, which is released from artificial substrate.

**Objective:** The objective of the work was to adapt a manual method to allow analysis of HEX in hemolyzed samples.

**Methods:** Serums without and with hemolysis were incubated with 4-nitrophenol-N-acetylglucosamine as a substrate. Released 4-nitrophenol was determined colorimetrically. After the incubation of the serum from hemolyzed blood with substrate, hemoglobin was precipitated with trichloroacetic acid (TCA) before 4-nitrophenol determination.

**Results:** The mean concentration of HEX activity in non-hemolyzed and hemolyzed blood of the same patients, determined with non-modified and modified methods had no significant differences, and they are: 243.12 ± 119.76 and 233.99 ± 108.76 pkat/mL, respectively. A coefficient of correlation between non-modified and modified methods equals the 0.98. For HEX determination with the modified method in serum from hemolyzed blood, optimal reaction time was 60 min, pH of reaction mixture was 4.7, and Km was 0.11 mMm.

**Conclusion:** HEX determinations in the same serums from non-hemolyzed blood by the non-modified method and hemolyzed blood with the modified method, gave similar results with a 0.98 coefficient of correlation. The modified method is appropriate for HEX determination in serum from hemolyzed blood.



## 1. Introduction

The activity of lysosomal exoglycosidases reflects the intensity of the degradation processes and has diagnostic value in medicine [1]. The most active of exoglycosidases, N-acetyl-β-hexosaminidase (HEX) [2] in urine and serum is a recognized marker of alcohol abuse [3], neoplasms [4], and inflammatory processes [5]. Determination of HEX and other exoglycosidases is based on quantification of the released 4-nitrophenol from appropriate artificial substrates by the exoglycosidase. Determination of HEX and other exoglycosidases in plasma and serum is limited by hemolysis. It is estimated that hemolyzed serum samples constitute of 10–30% all blood samples delivered to the routine clinical laboratory [6]. Blood hemolysis may be a result of pre-laboratory errors caused by improper collection, processing, or transport of the blood to the laboratory [7]. In vivo hemolysis occurs in some genetical diseases e.g. sickle cell anemia [8], autoimmune hemolytic anemia [9], beta-thalassemia [10], as well as mechanical damage of erythrocytes (e.g. by artificial cardial valve) [11], hemolysis uremic syndrome [12], pre-eclampsia [13], or paroxysmal nocturnal hemoglobinuria [14]. In forensic medicine the hemolyzed blood is the only diagnostic blood material [15]. In veterinary medicine there is a problem with the determination of exoglycosidases because of hemolysis in blood serum collected from stressed animals [16].

Because of the diagnostic importance of exoglycosidases in plasma and serum from hemolyzed blood, the aim of the present investigation

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was to elaborate a method for determination of HEX (the most active of exoglycosidases) in serum from hemolyzed human blood.

## 2. Materials and methods

### 2.1. Ethics

The research protocol was approved by the Senate Committee of Ethics for Scientific Research at the College of Computer Science and Business Administration, Łomża, Poland (protocol number: 4/2013/13.11.2013).

### 2.2. Blood collection

Blood was collected from the cubital vein into two test tubes from 70 volunteers (32 men and 38 women) aged 16–50 years (±35.5 years). The blood in the first test tube was allowed to clot in a typical way and in the second test tube blood was hemolyzed by vigorous shaking in 0.5–1 min, and then left for 30–60 min at a laboratory temperature until clot formation. Hemolyzed and non-hemolyzed blood samples were centrifuged for 10 min at 4000 rpm and +4 °C, in a laboratory centrifuge (MPW-350R, MPW Medical Instruments, Warsaw, Poland). The hemolyzed and non-hemolyzed serums were drawn off into Eppendorf type tubes and stored at −80 °C until enzymatic determinations.

### 2.3. Determination of the HEX activity in serum from non-hemolyzed blood

The substrate for determination of the HEX activity was 4-nitrophenyl-N-acetyl-β-glucosaminide (Sigma-Aldrich, St. Louis, USA). The calibrant of 4-nitrophenol (Sigma-Aldrich, St. Louis, USA) was prepared at concentration 0.25 mmol/L in a 100 mmol/L citrate–phosphate buffer of pH 4.7. Determination of the HEX activity in human serum without hemolysis was performed in duplicates by the colorimetric Marciniak et al. method [17] as follows: to 2 mL test tubes of the Eppendorf type were added: 50 μL of serum, 200 μL of 0.1 mol/L of Mc Ilvaine phosphate–citrate buffer pH 4.7, and 150 μL 6.7 mmol/L of 4-nitrophenyl-N-acetyl-β-glucosaminide, as a substrate. Then test tube contents were mixed and test tubes immersed in a constant climate chamber (Memmert HPP 110, Memmert GmbH + Co. KG, Germany), at 37 °C for 60 min. Enzymatic reaction was terminated by adding 1000 μL of a 0.2 mol/L borate buffer at pH 9.8. Then 280 μL of the reaction mixture was transferred to a microplate (96 well U Transparent, Greiner Bio One, Germany). The amount of released 4-nitrophenol was measured at 410 nm in colorimeter (Infinite® 200 PRO, TECAN, Switzerland) twice: first against reagent blank (10 μL distilled water + 70 μL 0.1 mol/L citrate–phosphate buffer pH 4.7 + 200 μL 0.2 mol/L borate buffer pH 9.8); secondly against serum blank (10 μL serum without hemolysis + 70 μL 0.1 mol/L citrate–phosphate buffer pH 4.7 + 200 μL 0.2 mol/L borate buffer pH 9.8). HEX activity was expressed in pkat/mL of serum.

### 2.4. Determination of the HEX activity in serum from hemolyzed blood

Determination of the HEX activity in human serum with hemolysis was performed in duplicates by the colorimetric method [17] as follows: to 2 mL test tubes of the Eppendorf type were added: 50 μL of serum, 200 μL 0.1 mol/L Mc Ilvaine phosphate–citrate buffer pH 4.7 and 150 μL 6.7 mmol/L of 4-nitrophenyl-N-acetyl-β-glucosaminide as a substrate, mixed and incubated in a climate chamber (Memmert HPP 110, Memmert GmbH + Co. KG, Germany) at 37 °C for 60 min. After incubation hemoglobin and other proteins were precipitated with 5 μL of saturated water solution of trichloroacetic acid (TCA) and centrifuged off for 5 min at 14,500 rpm (Microcentrifuge MiniSpin Plus, Eppendorf AG, Hamburg, Germany) at laboratory temperature. Then 80 μL of supernatant was transferred to a microplate (96 well U Transparent, Greiner Bio One, Germany) and 200 μL of a 0.4 mol/L borate buffer pH 9.8 was added. Amount of released 4-nitrophenol was measured at 410 nm in colorimeter (Infinite® 200 PRO, TECAN, Switzerland) twice: firstly — against reagent mixture (10 μL distilled water + 70 μL 0.1 mol/L citrate–phosphate buffer pH 4.7 + 200 μL 0.2 mol/L borate buffer pH 9.8); secondly, against hemolyzed serum blank (10 μL serum from hemolyzed blood after precipitation of hemoglobin and proteins with saturated TCA + 70 μL 0.1 mol/L citrate–phosphate buffer pH 4.7 + 200 μL 0.2 mol/L borate buffer pH 9.8). HEX activity was expressed in pkat/mL of serum.

### 2.5. Determination of hemoglobin in hemolyzed serum [18]

To 500 μL Drabkin reagent (Aqua-Med ZPAM Kolasa Sp. j. Łódź, Poland) was added 50 μL serum from hemolyzed blood or standard hemoglobin solution (Aqua-Med ZPAM Kolasa Sp. j. Łódź, Poland), mixed and left for 20 min. The absorbance of serum from hemolyzed blood was read with Drabkin reagent against Drabkin reagent with water (500 + 50 μL) at 540 nm in colorimeter Infinite® 200 PRO, TECAN, Switzerland.

### 2.6. Determination of maximum hemoglobin absorbance in incubation mixtures

a) Without precipitation of hemoglobin with TCA — to the microplate well was added: 10 μL of serum with hemolysis + 70 μL 0.1 mol/L citrate–phosphate buffer + 200 μL 0.2 mol/L borate buffer pH 9.8.
b) After precipitation of hemoglobin with TCA — to a 1.5 mL test tube of Eppendorf type was added: 50 μL of serum with hemolysis + 350 μL of 0.1 mol/L citrate–phosphate buffer + 5 μL of saturated TCA and centrifuged for 5 min at 14,500 rpm in the Microcentrifuge MiniSpin Plus, Eppendorf AG, Hamburg, Germany, at laboratory temperature. To 80 μL of supernatant was added 200 μL 0.2 mol/L borate buffer pH 9.8.

Absorbances of incubation mixtures were scanned with hemolyzed serum without (a) and after hemoglobin precipitation (b) from 380 to 460 nm at 5 nm intervals against reagent blank (10 μL of distilled water + 70 μL 0.1 mol/L citrate–phosphate buffer + 200 μL 0.2 mol/L borate buffer pH 9.8) at colorimeter Infinite® 200 PRO, TECAN, Switzerland.

### 2.7. Statistical analysis

The collected data were analyzed statistically using Statistica version 10.0 (Statsoft, Cracow, Poland) with the Student's test and Pearson's correlation coefficients. A value of $p < 0.05$ was taken as being significant.

## 3. Results

### 3.1. Hemoglobin in hemolyzed blood serum

In our samples of serum from hemolyzed human blood was ranged 0.24–3.47 g/dL (mean 1.10 ± 1,28 g/dL) of hemoglobin. In the absorbance scan of serum from hemolyzed blood (Fig. 1) one can see a peak of absorbance at 415 nm that was removed by precipitation of hemoglobin by TCA treatment.

### 3.2. Characterization of HEX activity in serum from hemolyzed blood

A Lineweaver–Burk plot for determination Km for HEX in serum from hemolyzed blood amounted to 0.11 mmol/L, and was very similar to the Km of the unmodified assay of 0.125 mmol/L. HEX in serum from hemolyzed blood was active at pH from 3.5–5.5 with an optimum at pH 4.6. We observed in an unmodified assay a very similar relation