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Original research article

Optimization of the method for α-L-fucosidase, β-D-galactosidase and β-D-glucuronidase determination in serum from hemolyzed blood

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ABSTRACT

Purpose: Adaptation of the colorimetric method for the determination of β-D-galactosidase, β-D-glucuronidase and α-L-fucosidase activities in serums from hemolyzed blood, the material currently being discarded.
Materials and Methods: The materials included serums from hemolyzed and non-hemolyzed blood, obtained from 26 healthy volunteers. The adaptation of the method involved precipitation of the proteins with trichloroacetic acid after incubating serums with substrates, but before determining the products of enzymatic reactions.
Results: In serums from hemolyzed and non-hemolyzed blood of the same persons, we found high correlations among the results obtained using hemolyzed blood (with adapted) and non-hemolyzed blood (with non-adapted) methods.
Conclusion: We are able to determine the β-D-galactosidase, β-D-glucuronidase and α-L-fucosidase activities in serums from hemolyzed blood (with adapted) and non-hemolyzed blood (with non-adapted) methods, with the same accuracy and precision.

1. Introduction

The activity of lysosomal exoglycosidases present in all cells and body fluids, with the exception of erythrocytes [1], reflects the degradation intensity of: glycoproteins and glycolipids (α-L-fucosidase - FUC) [2], glycoproteins, glycolipids and keratan sulfate (β-D-galactosidase - GAL) [3] and glycosaminoglycans (β-D-glucuronidase – GLU) [4,5]. Therefore, determining the activity of particular exoglycosidases in the body fluids may be used for diagnosiing and monitoring the diseases that involve increased catabolism of appropriate glycoconjugates [6–11]. The available research recommend using increased serum GAL activity as a marker of colon [12] and larynx cancers [13]. Serum GLU activity could be a useful marker of hepatitis progression [14] and increased degradation of proteoglycans in diabetes [15]. The increase in serum FUC in patients with liver cirrhosis and small tumors of hepatocellular carcinoma is a promising marker of both aforementioned diseases, especially since the currently used markers (α-fetoprotein and des-γ-carboxy-prothrombin) seem to be less specific in detecting hepatocellular carcinoma than previously expected [16–19].

Determination of serum exoglycosidases, based on quantification at 410 nm, of released 4-nitrophenol from appropriate artificial substrates may be strongly affected by the presence of hemoglobin released by damaged erythrocytes, which show maximum absorbance at 415, 540 and 570 nm [20].

Hemolysis is responsible for rejecting nearly 60% of serums in routine diagnostic laboratories [21,22]. Hemolysis may result from pre-laboratory errors: improper collecting, processing, or transporting of the blood samples to the laboratory [20]. A serious problem arises with in vivo hemolysis that occurs in forensic medicine [23] and genetic diseases, such as: sickle cell anemia [24], autoimmune hemolytic anemia [25], beta-thalassemia [26] and hemolytic uremic syndrome [27], preeclampsia [28], and paroxysmal nocturnal hemoglobinuria [29].

The aim of the present study was to optimize and validate the Marciniak et al. method [5] for determining GAL, GLU and FUC in serum from hemolyzed blood, based on our previous experience [30,31].

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Table 1
Hemoglobin concentration in 26 serum samples from hemolyzed blood.

donor	hemoglobin concentration [g/dL]
1	0.4
2	0.5
3	0.6
4	0.3
5	0.3
6	0.3
7	0.3
8	0.3
9	0.4
10	0.3
11	0.3
12	0.4
13	0.3
14	0.6
15	0.3
16	0.4
17	0.3
18	0.3
19	0.5
20	0.3
21	0.3
22	0.6
23	0.4
24	0.9
25	0.7
26	0.5
mean ± SD	0.4 (0.3–0.9) ± 0.1

2. Materials and methods

2.1. Ethics

The research was approved by the Ethics Committee at Lomza State University of Applied Sciences, Łomża, Poland (protocol: 4/2013/13.11.2013).

2.2. Materials

Substrates and 4-nitrophenol were from Sigma, St. Louis, MO, USA. We used the following substrates: 4-nitrophenyl-β-D-galactopyranoside (6.7 mM in 0.1 M phosphate-citrate buffer pH 4.3) for GAL determination; 4-nitrophenyl-α-L-fucopyranoside (6.7 mM in 0.1 M phosphate-citrate buffer pH 4.3) for FUC determination; 4-nitrophenyl-β-D-glucuronide (6.3 in 0.1 M acetate buffer pH 4.5 (for GLU determination) and 4-nitrophenol standard (0.3 mM). We used the following buffers: Mc Ilvaine phosphate-citrate 0.1 M pH 4.3 (for FUC and GAL determination), acetate 0.1 M pH 4.5 (for GLU determination) and borate 0.2 and 0.4 M, pH 9.8. Drabkin reagent and hemoglobin standard were from Aqua-Med ZPAM Kolasa Sp. j. Łódź, Poland.

2.3. Blood collection

Blood was collected from the cubital vein (S-Monovette®, SARST-EDT, Poland) into two test tubes from 26 healthy volunteers (16 males and 10 females) aged 23–55 years (mean 36), who came to the laboratory for prophylactic examinations. One of the samples was hemolyzed by vigorous shaking (0.5–1 min), and the second was protected from hemolysis. Both samples were left (30–60 min.) at a room temperature (RT), centrifuged (10 min, 6000 × *g* at + 4 °C (MPW-

Table 2
Small differences in exoglycosidases activity [nKat/L] in the same serums without and with hemolysis obtained from 26 donors.

donor	FUC		GAL		GLU	
	Serum					
	without	with	without	with	without	with
	hemolysis					
1	81.6	77.9	20.4	20.2	20.4	20.2
2	49.3	47.8	23.6	24.2	23.6	24.2
3	42.7	48.9	22.0	21.3	22.0	21.3
4	96.7	93.7	26.7	26.3	26.7	26.3
5	77.0	74.4	29.8	28.0	29.8	28.0
6	72.8	73.3	13.4	13.4	13.4	13.4
7	86.7	79.6	12.0	15.3	12.0	15.3
8	89.9	65.6	28.9	23.4	28.9	23.4
9	38.5	41.5	15.3	16.9	15.3	16.9
10	50.8	44.0	21.4	20.9	21.4	20.9
11	37.8	34.9	19.4	16.9	19.4	16.9
12	74.6	78.3	11.7	13.3	11.7	13.3
13	40.8	64.9	15.8	15.6	15.8	15.6
14	93.3	80.4	38.9	36.9	38.9	36.9
15	29.9	28.5	5.9	5.9	48.5	43.4
16	24.8	25.1	6.6	7.5	65.3	64.9
17	28.0	23.1	4.7	4.9	89.2	99.5
18	32.8	21.8	11.1	10.9	93.6	94.2
19	28.2	27.8	14.9	15.9	83.3	80.5
20	43.5	42.8	17.9	18.8	16.0	17.9
21	46.8	45.9	7.7	7.7	42.7	37.3
22	33.5	33.8	22.8	23.8	47.7	51.8
23	36.9	33.8	6.9	7.9	34.4	40.7
24	14.6	12.6	9.9	10.9	39.4	46.3
25	23.7	24.2	9.9	10.7	25.5	28.3
26	16.9	17.7	12,1	13.2	74.8	72.3
mean ± SD	49.7 ± 24.9	47.8 ± 23.1	16.5 ± 8.5	16.6 ± 7.7	36.9 ± 24.8	37.3 ± 25.4
coefficient of correlation	**0.99**		**0.98**		**0.99**	

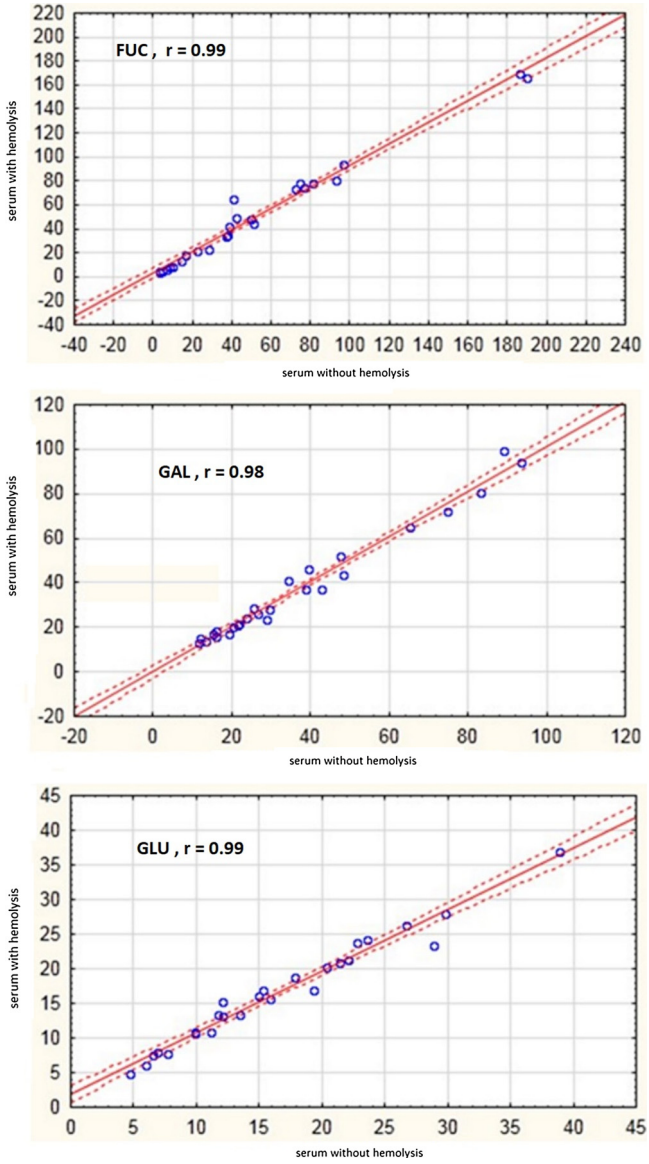

Fig. 1. Correlations of exoglycosidases activity between the same person's serums with and without hemolysis.

Table 3
Precisions of exoglycosidases determination in the same serum from hemolyzed blood.

Precision		
lysosomal exoglycosidase	[nKat/L]	CV
within run		
FUC	41.3 ± 1.6	8.0%
GAL	10.7 ± 0.7	3.4%
GLU	15.9 ± 0.5	2.6%
day to day		
FUC	40.9 ± 0.8	9.0%
GAL	10.6 ± 0.8	3.8%
GLU	16.0 ± 0.4	2.2%

350R, MPW Medical Instruments, Warsaw, Poland), transferred to plastic tubes, and stored (−80 °C) until used (2 weeks).

2.4. Determining serum hemoglobin concentration

The serum hemoglobin concentration was determined according to the Drabkin method [32].

2.5. Determining FUC, GAL, and GLU activities in serums from non-hemolyzed blood [5]

10 µL of serum, 40 µL of Mc Ilvaine phosphate-citrate buffer (for FUC and GAL determination) or acetate buffer (for GLU determination), and 30 µL of adequate substrates were mixed in a 96-well microplate. Samples were incubated (60 min. in 37 °C) with constant shaking and the reaction was terminated by 200 µL of 0.2 M borate buffer. The released 4-nitrophenol was measured at 410 nm (Infinite® 200 PRO, TECAN, Switzerland) twice: first, against reaction mixture without serum, then serum in reaction mixture without substrate. FUC, GAL and GLU activities were expressed in nKat/L of serum.

Optimal conditions for FUC, GAL, and GLU determination in serum from non-hemolyzed blood were chosen as follows:

- Km: 0.5–2.5 mM substrate concentrations at pH 4.3 (for FUC and GAL determination), and pH 4.5 (for GLU determination), and incubation for 60 min at 37 °C for all lysosomal exoglycosidases;
- incubation time: 20, 40, 60, 80, 100 and 120 min. with a substrate concentration of 6.7 mM (for FUC and GAL determination), and 6.3 mM (for GLU determination), and pH 4.3 (for FUC and GAL determination) and pH 4.5 (for GLU determination);
- effect of pH: pH 3.9–4.9 with a substrate concentration of 6.7 mM (for FUC and GAL determination), and 6.3 mM (for GLU determination), and the incubation time of 60 min at 37 °C.

2.6. Determining FUC, GAL, and GLU activities in serums from hemolyzed blood

To 50 µL of serums from hemolyzed blood in 2 mL plastic tubes we added, 200 µL of phosphate-citrate buffer to determine FUC and GAL or 200 µL of acetate buffer to determine GLU, and 150 µL of adequate substrate in buffer. The contents were then mixed, and incubated (60 min., 37 °C). The proteins were precipitated by saturated (at RT) water solution of trichloroacetic acid (TCA) (5 µL) and microcentrifuged (5 min., 14 000 × *g* at RT). Supernatants (80 µL) were transferred to a microplate, and borate buffer (0.4 M, 200 µL) was added. Released 4-nitrophenol was measured at 410 nm (Infinite® 200 PRO, TECAN, Switzerland) twice: first, against the reaction mixture without serum, and then against serum from hemolyzed blood in the reaction mixture without substrate. Exoglycosidase activities were expressed in nKat/L of serum.

Optimal conditions for determining FUC, GAL, and GLU in serum from hemolyzed blood were chosen as follows:

- Km were determined at substrate concentrations of 0.5–2.5 mM at pH 4.3 (for determining FUC and GAL) or pH 4.5 (for determining GLU) after 60 min. of incubation at 37 °C;
- Incubation time: 20, 40, 60, 80, 100 and 120 min. incubations were tested at substrate concentration 6.7 mM (for determining FUC and GAL) and 6.3 mM (for determining GLU), with pH 4.3 (for determining FUC and GAL) and pH 4;5 (for determining GLU).
- The effects of pH were determined at pH 3.9–4.9 with substrate concentrations of 6.7 mM (for determining FUC and GAL) and 6.3 mM (for determining GLU) after 60 min. of incubation at 37 °C.

2.7. Precision within run and day to day

Precisions within run (twenty times on the same day) and day to day (once a day for 20 consecutive days) for FUC, GAL and GLU activities

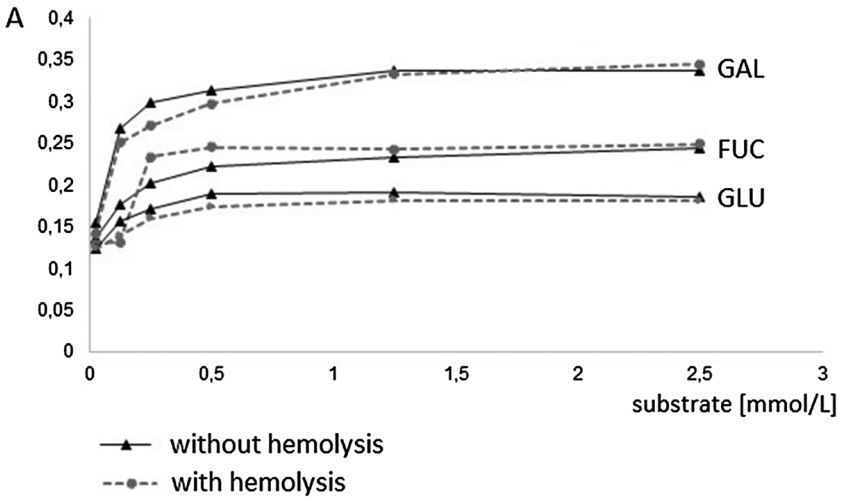

Fig. 2. Michaelis-Menten plots for the same person's exoglycosidases activity in serums with and without hemolysis.

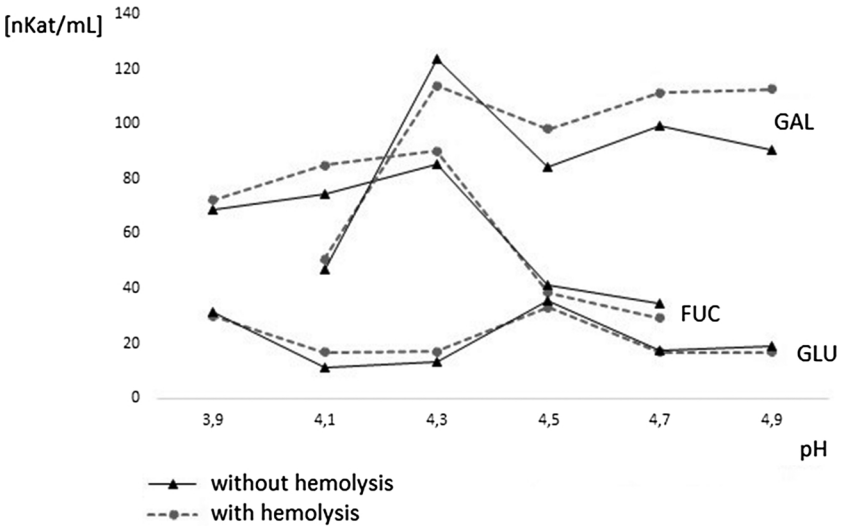

Fig. 3. Effect of pH on the same person's exoglycosidases activity in serums with and without hemolysis.

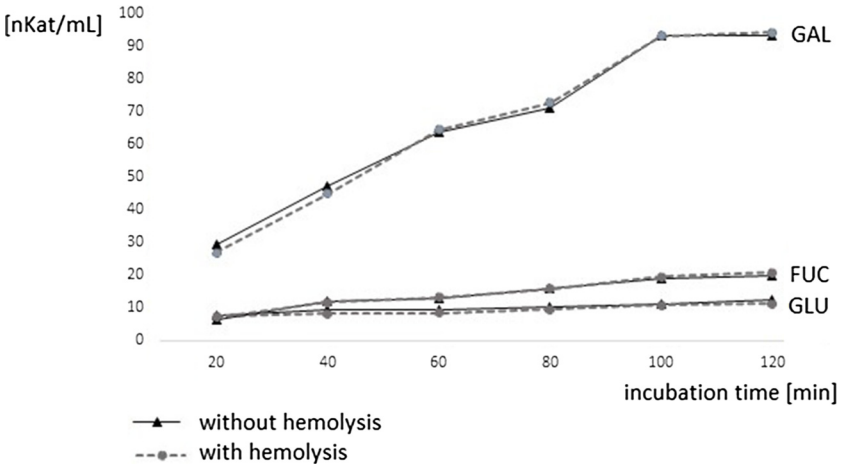

Fig. 4. Effect of incubation time on 4-nitrophenol released by the same person's serum with and without hemolysis.

were determined on the same serum from hemolyzed blood sample, as described in Section 2.6.

2.8. Statistical analysis

The data was analyzed statistically using Statistica version 10.0 (Statsoft, Cracow, Poland) with the Student's *t*-test and Pearson's correlation coefficients. A value of $p < 0.05$ was taken as statistically significant.

3. Results and discussion

This paper concerns FUC, GAL and GLU activities in serum from hemolyzed blood based on the determination of released 4-nitrophenol concentration at 410 mn. Determining the 4-nitrophenol concentration is considerably affected by the presence of hemoglobin [20]. The upper limit of the reference range for serum free of hemoglobin is 0.05 g/dL. When hemoglobin serum concentration is above 0.2 g/L, the serum has a visible pink hue, and above 0.6 g/dL, it is distinctly red [33]. Our serums from hemolyzed blood were visibly pink-red and they contained from 0.3 to 0.9 (0.4 ± 0.1) g/dL of hemoglobin (Table 1).

We were not able to determine FUC, GAL, and GLU activities with the Marciniak et al. method [5] in our serum samples from hemolyzed blood using one or two blanks (substrate without serum and serum without substrate) because E_{410} of the incubation mixtures were too large (greater than 3.00). We precipitated serum proteins, including hemoglobin with TCA, based on our experience in determining *N*-acetyl-β-hexosaminidase [30]. Previously, we stated that scans of the same person's serum from hemolyzed blood with TCA treatment, and non-hemolyzed blood without TCA treatment were very similar [30]. The FUC, GAL and GLU activities in the same 26 persons' serum samples with and without hemolysis (determined with modified and non-modified method, respectively) were: 47.8 ± 23.1 nKat/L and 49.7 ± 24.9 nKat/L (for FUC); 16.6 ± 7.7 nKat/L and 16.5 ± 8.2 nKat/L (for GAL); 37.3 ± 25.4 nKat/L and 36.9 ± 24.8 nKat/L (for GLU) (Table 2), with correlation of the coefficients 0.99 (for FUC), 0.98 (for GAL) and 0.99 (for GLU) (Fig. 1), standard error of the estimation: 7.3, 1.5 and 3.7 for FUC, GAL and GLU. For FUC, GAL and GLU determination in serums from hemolyzed bloods with TCA treatment, coefficients of variation amounted: for precision within run: 8.0%, 3.4%, 2.6%, and for precision day to day: 9.0%, 3.8%, 2.2% (Table 3), respectively. The above results present the proof that the proposed adaptation of the Marciniak et al. method [5] is reproducible and repetitive. Furthermore, we are currently sure that the storage time of the samples does not affect the activity of lysosomal exoglycosidases in hemolyzed blood because the results obtained in the alternate 20 days do not show any differences.

In hemolyzed and non-hemolyzed blood serums of the same person: Km for FUC, GAL, and GLU (Fig. 2) were similar and amounted to 0.2 M (for FUC), 0.12 mM (for GAL) and 0.2 mM (for GLU); FUC, GAL and GLU (Fig. 3) were active at pH from 3.9 to 4.9 with an optimum at pH 4.3 (for FUC & GAL) and 4.5 (for GLU); amounts of 4-nitrophenol released by FUC, GAL and GLU during 20-120 min. incubations of the same person's serum from non-hemolyzed and hemolyzed blood were similar (Fig. 4). To obtain optimal reading at 410 nm we chose 60 min. for serum of hemolyzed blood.

We found a large dispersion of results within the study group (Table 2). Considerable differences in the results of FUC, GAL and GLU activity in serums of individual persons may be caused by differences in age, sex, and health conditions of persons included in our study group. Our volunteers declared good health, but their statements were not verified by medical examination. It is worthy to note that the observed differences in FUC, GAL and GLU serum activities of individual persons, may be the subject of a separate paper, but are not important in the case of our experiment because our goal was to check the correctness of the modified method.

4. Conclusion

Our modification of the Marciniak et al. method [5] make precise determination of FUC, GAL and GLU activities possible for the cases where it is impossible to obtain serum without hemolysis (e.g. genetical diseases, post mortem examinations) or when the blood is hemolyzed during pre-laboratory procedure.

Conflict of interest

The authors declare no conflict of interests.

Financial Disclosure

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