Mashhad University of Medical Sciences (MUMS)

Reviews in Clinical Medicine

Clinical Research Development Center Ghaem Hospital

Validation and standardization of a fluorometric method for alpha glucosidase activity assay in the detection of Pompe

*Armin Mokhtariye (PhD Student)[1,2], Lida Hagh-Nazari (PhD)[2], Abdol-Reza Varasteh (PhD)[3,4], Hadi Mozafari (PhD)[2], Fatemeh Keyfi (PhD)[4]**

[1]Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.
[2]Department of Clinical Biochemistry, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran.
[3]Immunobiochemistry Lab, Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Department of Medical Laboratory Sciences, Varastegan Institute for Medical Sciences, Mashhad, Iran

ARTICLE INFO

Article type
Original article

Article history
Received: 24 Apr 2023
Revised: 16 May 2023
Accepted: 6 Jun 2023

Keywords
Lysosomal Storage Disease
Pompe, Fluorometric method
Enzyme Activity Assay

ABSTRACT

Introduction: Pompe Disease is a type of lysosomal storage disease that is caused by a deficiency of the lysosomal alpha glucosidase. Pompe disease, as a multi-systemic disorder has a broad spectrum of clinical symptoms. The objective of this research was to validate and standardize the fluorometric method to detect alpha glucosidase activity, which can be used to identify patients with Pompe disease.

Methods: This study was performed on 45 Pompe patients and 50 healthy control subjects. Dried blot spots were collected and subjected to an alpha glucosidase activity assay using the fluorometric method. The obtained fluorometric outcomes were then compared to those of the validated MS-MS method.

Results: The control group showed a greater level of lysosomal alpha glucosidase activity compared to patients with Pompe disease (P value <0.001). A strong correlation was observed between fluorometric and MS-MS methods, as indicated by the high correlation coefficient (R0.955=2).

Conclusion: Accurate and reliable detection of Pompe disease is based on laboratory diagnosis. Alpha glucosidase activity assays are used for initial diagnosis because of they are cost-effective and simple. However, the current research shows that the fluorometric method is also a reliable, cost-effective, and simple alternative to identify Pompe disease.

Please cite this paper as:
Mokhtariye A, Hagh-Nazari L, Varasteh A, Mozafari H, Keyfi F. Validation and standardization of a fluorometric method for alpha glucosidase activity assay in the detection of Pompe disease. Rev Clin Med. 2023;10 (2): 22-26.

Introduction

Pompe Disease (PD) is a type of lysosomal storage disease. PD is caused by a deficiency of the lysosomal alpha glucosidase (GAA), which results in the accumulation of glycogen in the lysosomes of skeletal and cardiac muscles. The frequency of PD is estimated to be one in every 40000 live births in the United States. PD is a multi-systemic disorder and has a wide range of clinical symptoms that vary depending on the age of onset and include cardiomegaly, hepatomegaly, macroglossia, etc. PD is divided into infantile-onset (severe) and late-onset (mild) clinical forms (1-5).

Clinical involvement in the severe infantile-onset form appears in the first weeks after birth with progressive hypertrophic cardiomyopathy and generalized muscular weakness, which leads to death due to cardiorespiratory failure. In individuals with the mild late-onset form, clinical symptoms emerge during their second or third decade of life. It should be noted that signs and

***Corresponding author:** Fatemeh Keyfi ,
Department of Medical Laboratory Sciences, Varastegan Institute for Medical Sciences, Mashhad, Iran.
E-mail: keyfi.fateme@gmail.com
Tel: 5135091160

symptoms are limited to muscular myopathy and cardiac dysfunction is not seen (1,6).

The GAA activity assay and molecular examination are standard tests used for laboratory diagnosis of Pompe patients (7,8). However, the GAA activity assay is not performed in Iran and DBSs are sent to other countries, resulting in prolonged detection time. Hence, the objective of this research was to validate and standardize the fluorometric method as an alternative to the GAA activity assay to accurately and easily detect alpha glucosidase activity to diagnosis Pompe patients.

Materials and Methods

Subjects and ethics

This study was performed on 45 infantile Pompe patients and 50 healthy control subjects. The inclusion criteria were: age<2 years, patients screened by the MS-MS method, and confirmation by an endocrinologist and metabolism specialist. Before conducting this study, the clinical and MS-MS enzymatic assay confirmed the diagnosis for all patients (9). The procedures followed in this study were consistent with the ethical standards set forth by the Committee on Human Experimentation at Kermanshah University of Medical Science (KUMS), as evidenced by project No. 95311.

Sampling

To obtain dried blot spots (DBS), a blood sample was collected in an EDTA tube and immediately spotted onto Whatman 903® specimen collection paper, which served as a filter paper. DBSs were stored at -20°C until analysis time.

Fluorometric assay for GAA in DBS

The GAA activity assay was performed by the fluorometric method (modified Chamoles method) (9,10). Briefly, a 3.2 mm punch from DBS was transferred to a microtube. Then 300µl of distilled water was added to it, and the microtube was sealed with plastic sealing film. Samples were shaken at 37°C using a shaker orbit at 250 rpm for one hour.

Next, microtubes were centrifuged at 3,000×g for two min. GAA activity was run by mixing 150µl of DBS extract, 250 µl artificial substrate 4-methylumbelliferyl-α-D-glucoside (1.4 mM, in 40 mM sodium acetate (NaCH3COO) buffer at pH 3.8), and 50µl of 80 µM acarbose solution. Following a 20-hour incubation period at 37°C, the reaction was stopped through the introduction of 1000 µl of EDTA buffer (0.5 M, pH 11.5). All tests were run in duplicate.A standard curve was employed for 4-methylumbelliferone, with concentrations ranging from 0 to 8 pmol, to determine enzyme activities. The calculations were based on this curve. The fluorescence was read on a Perkin-Elmer LS45 instrument (em: 360 nm, ex: 450 nm). GAA activity was reported by µmol/h/liter.

Fluorometric assay for beta galactosidase in DBS

The beta galactosidase (BGAL) activity assay was performed by the fluorometric method as the external control. For DBS extract, 200 µl of citrate phosphate buffer (0.1 M, pH=5) and 200 µl of sodium chloride (0.9%) were added to a microtube, and 3.2 mm punch from DBS was transferred to it. Microtubes were shaken at 25°C using a shaker orbital at 250 rpm for one hour. Then microtubes were centrifuges at 3000×g for two min. BGAL activity was run by mixing 200 µl of DBS extract, 150 µl of artificial substrate 4-methylumbelliferyl-β-D-galactopyranoside (0.8 mM).

After incubation for 20 hours at 37°C, the reaction was stopped by the addition of 200µl EDTA buffer (0.5 M, pH 11.5). All tests were run in duplicate. We used a standard curve for 4-methylumbelliferone, and contained concentrations of 0 to 8 pmol, which was used for the calculation of enzyme activities. The fluorescence was read on a Perkin-Elmer LS45 instrument (em: 360nm, ex: 450 nm). BGAL activity was reported by µmol/h/liter.

Results

Characteristics of GAA and BGAL by the fluorometric method

Characteristics and the laboratory data for GAA and BGAL activity are presented in Table 1.

Table1. Characteristics of the alpha glucosidase (GAA) activity assay and beta galactosidase (BGAL) activity assay in our study population

Parameter	GAA activity (µmol/h/liter)		P value	BGAL activity (µmol/h/liter)		P value
	PD patients (n=45)	Controls (n=50)		PD patients (n=25)	Controls (n=50)	
Sex (males)	23	25	>0.05	16	25	>0.05
Age (years)	1.9+0.5	2.1+0.3	>0.05	1.9+0.5	2.1+0.3	>0.05
Average of enzyme activity	0.78	4.1	<0.001	4.5	4.5	>0.05
Minimum of enzyme activity	0.11	2.0	<0.001	3.3	3.2	>0.05
Maximum of enzyme activity	2.7	6.2	<0.001	5.5	6.1	>0.05
Cut-off	2.0		-	-		-

The control group showed a greater level of GAA activity compared to patients with PD (P value <0.001). BGAL activity has no significant difference in both groups (P value >0.05). Dot plot curve for GAA activity assay in PD patients and control group are shown in Figure 1.

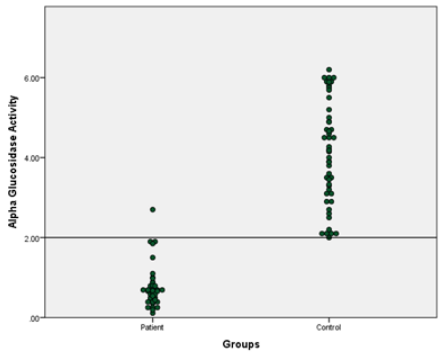

Figure 1. Dot plot curve for GAA activity assay

Standardization and validation of the GAA activity assay by the fluorometric method

ROC curve for the fluorometric GAA activity assay is shown in Fig 2. GAA activity had %100 sensitivity and %98.4 specificity

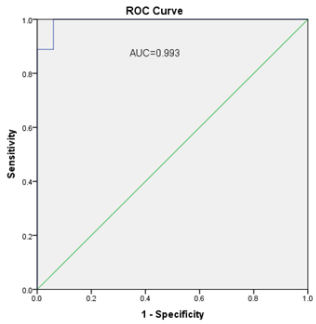

Figure 2. ROC curve for GAA activity (AUC=area under curve)

The coefficient of variation (CV) in between run and within run was 12.3% and 3.1%, respectively. Also, accuracy was 98.6%.

Linear regression of GAA activity for comparison between fluorometric and MS-MS methods are presented in Fig 3. A strong correlation was observed between the methods, as indicated by the high correlation coefficient (R2=0.955).

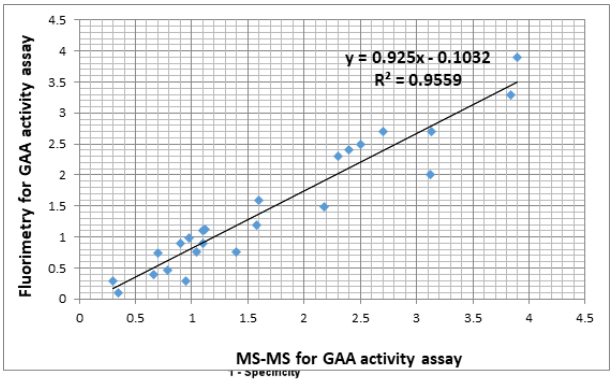

Figure 3. Regression curve for GAA activity to compare fluorometric and MS-MS methods

Discussion

In Pompe disease, clinical manifestations not only are varied and non-specific, but are also overlapping with other metabolic disorders, making the diagnosis of Pompe disease challenging (11-15).

Hence, laboratory diagnosis is crucial to precisely diagnosis patients with PD. Alpha glucosidase activity assays are commonly utilized as a primary diagnostic tool and are typically incorporated into newborn screening programs because they are cost-effective and simple (8). Early diagnosis and timely treatment for Pompe patients is important because it can reduce some irreversible damage and improve their quality of life (16,17). The GAA activity assay is not performed in Iran and DBSs are sent to

other countries, resulting in long detection time. This is the first study performed on an Iranian population in which validation and standardization of the alpha glucosidase activity assay by the fluorometric method as a simpler and cost-effective method has been studied (18).

In a study done by Chamoles et al., no overlap between Pompe patients and their control group were reported, although they did not sperate the carrier and control groups based on the fluorometric method (6). Other studies have had good differentiate between their control groups and Pompe groups. Our study is in agreement with these results (19-21).

Our results showed a high correlation between fluorometric and MS-MS methods for GAA activity. Chieh Liao et al. obtained similar results with the present study. In addition, they also examined Pompe disease carriers and pseudodeficiency newborns and found that the fluorometric assay separated <10% of the pseudodeficiencies from the homozygote Pompe patients (22).

Also, Musumeci et al. used both the fluorometric and MS-MS assay for newborn screening, although overlap between these methods was not complete (23). According to Elbin et al., reported GAA activity was not the same between fluorometric and MS-MS methods, although the effect of pre-analytic variables (such as blood volume, etc.) had similar effects on GAA activity in both methods (24).

Conclusion

The current research shows that the fluorometric method is a reliable marker for identifying Pompe disease. Hence, it has the potential to be utilized in newborn screening.

Acknowledgment

We gratefully acknowledge the support given to us by the Kermanshah University of Medical Science, and we also thank the Varastegan Institute for Medical Science, Mashhad, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Bodamer OA, Dajnoki A. Diagnosing lysosomal storage disorders: Pompe disease. Current protocols in human genetics / editorial board, Jonathan L Haines [et al]. 2012;Chapter 17:Unit17.1.
2. Ebrahimi M, Behnam M, Behranvand-Jazi N, Yari L, Sheikh-Kanlomilan S, Salehi M, et al. Identification a novel mononucleotide deletion mutation in GAA in pompe disease patients. Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences. 2017;22.
3. Kishnani PS, Amartino HM, Lindberg C, Miller TM, Wilson A, Keutzer J. Methods of diagnosis of patients with Pompe disease: Data from the Pompe Registry. Molecular genetics and metabolism. 2014;113(1-2):84-91.
4. Er TK, Chen CC, Chien YH, Liang WC, Kan TM, Jong YJ. Development of a feasible assay for the detection of GAA mutations in patients with Pompe disease. Clinica chimica acta; international journal of clinical chemistry. 2014;429:18-25.
5. McIntosh P, Austin S, Sullivan J, Bailey L, Bailey C, Viskochil D, et al. Three cases of multi-generational Pompe disease: Are current practices missing diagnostic and treatment opportunities? American Journal of Medical Genetics Part A. 2017;173(10):2628-34.
6. Chamoles NA, Niizawa G, Blanco M, Gaggioli D, Casentini C. Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper. Clinica chimica acta. 2004;347(1-2):97-102.
7. Ceci R, Francesco PNd, Mucci JM, Cancelarich LN, Fossati CA, Rozenfeld PA. Reliability of enzyme assays in dried blood spots for diagnosis of 4 lysosomal storage disorders. Advances in Biological Chemistry. 2011;Vol.01No.03:7.
8. Mokhtariye A, Hagh-Nazari L, Varasteh A-R, Keyfi F. Diagnostic methods for Lysosomal storage disease. Reports of biochemistry & molecular biology. 2019;7(2):119.
9. Chamoles NA, Blanco M, Gaggioli D, Casentini C. Gaucher and Niemann-Pick diseases--enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn-screening cards. Clinica chimica acta; international journal of clinical chemistry. 2002;317(1-2):191-7.
10. Lukacs Z, Nieves Cobos P, Mengel E, Hartung R, Beck M, Deschauer M, et al. Diagnostic efficacy of the fluorometric determination of enzyme activity for Pompe disease from dried blood specimens compared with lymphocytes—possibility for newborn screening. Journal of Inherited Metabolic Disease: Official Journal of the Society for the Study of Inborn Errors of Metabolism. 2010;33(1):43-50.
11. Zimran A, Belmatoug N, Bembi B, Deegan P, Elstein D, Fernandez-Sasso D, et al. Demographics and patient characteristics of 1209 patients with Gaucher Disease: Descriptive analysis from the Gaucher Outcome Survey (GOS). American Journal of Hematology. 2017.
12. Devigili G, De Filippo M, Ciana G, Dardis A, Lettieri C, Rinaldo S, et al. Chronic pain in Gaucher disease: skeletal or neuropathic origin? Orphanet journal of rare diseases. 2017;12(1):148.
13. Almeida V, Conceição I, Fineza I, Coelho T, Silveira F, Santos M, et al. Screening for Pompe disease in a Portuguese high risk population. Neuromuscular Disorders. 2017.
14. Kishnani PS, Steiner RD, Bali D, Berger K, Byrne BJ, Case LE, et al. Pompe disease diagnosis and management guideline. Genetics in Medicine. 2006;8(5):267.
15. Manganelli F, Ruggiero L. Clinical features of Pompe disease. Acta Myologica. 2013;32(2):82.
16. Andrade-Campos M, Alfonso P, Irun P, Armstrong J, Calvo C, Dalmau J, et al. Diagnosis features of pediatric Gaucher disease patients in the era of enzymatic therapy, a national-base study from the Spanish Registry of Gaucher Disease. Orphanet Journal of Rare Diseases. 2017;12:84.
17. van Dussen L, Biegstraaten M, Hollak CE, Dijkgraaf MG. Cost-effectiveness of enzyme replacement therapy for type 1 Gaucher disease. Orphanet J Rare Dis. 2014;9:51.
18. Keyfi F, Nasseri M, Nayerabadi S, Alaei A, Mokhtariye A, Varasteh A. Frequency of inborn errors of metabolism in a northeastern Iranian sample with high consanguinity rates. Human heredity. 2018;83(2):71-8.
19. Sista RS, Eckhardt AE, Wang T, Graham C, Rouse JL, Norton SM, et al. Digital microfluidic platform for multiplexing enzyme assays: implications for lysosomal storage disease screening in newborns. Clinical chemistry. 2011;57(10):1444-51.
20. Tehrani KHN, Sakhaeyan E, Sakhaeyan E. Evaluation prevalence of Pompe disease in Iranian patients with myopathies of unknown etiology. Electronic physician. 2017;9(7):4886-9.
21. Sista RS, Wang T, Wu N, Graham C, Eckhardt A, Winger T, et al. Multiplex newborn screening for Pompe, Fabry, Hunter, Gaucher, and Hurler diseases using a digital microfluidic platform. Clinica chimica acta; international journal of clinical

chemistry. 2013;424:12-8.
22. Liao HC, Chan MJ, Yang CF, Chiang CC, Niu DM, Huang CK, et al. Mass Spectrometry but Not Fluorimetry Distinguishes Affected and Pseudodeficiency Patients in Newborn Screening for Pompe Disease. Clinical chemistry. 2017;63(7):1271-7.
23. Musumeci O, la Marca G, Spada M, Mondello S, Danesino C, Comi GP, et al. LOPED study: looking for an early diagnosis in a late-onset Pompe disease high-risk population. 2016;87(1):5-11.
24. Elbin CS, Olivova P, Marashio CA, Cooper SK, Cullen E, Keutzer JM, et al. The effect of preparation, storage and shipping of dried blood spots on the activity of five lysosomal enzymes. Clinica chimica acta; international journal of clinical chemistry. 2011;412(13-14):1207-12.