VALIDATION OF BETA-GLUCOSIDASE ACTIVITY ASSAY IN LEUKOCYTES

by

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A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Laboratory Medicine and Biomedical Science

Department of Pathology

The University of Utah

May 2016

The University of Utah Graduate School

STATEMENT OF THESIS APPROVAL

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ABSTRACT

Gaucher disease (GD) is an autosomal recessive disorder caused by deficiency of the lysosomal hydrolase glucocerebrosidase (GBA) (EC 3.2.1.45), required for the degradation of glycosphingolipids. In Gaucher disease, as a result of GBA deficiency, the body is unable to breakdown glucocerebroside, thus allowing the accumulation of glucosylceramide and glucosphingosine within the lysosomal cells. The clinical presentation of GD consists in a multisystem disorder, and its classification of GD into its three subtypes is dependent primarily on the presence or absence of neurological features. Gaucher disease type 1 is the most common inherited genetic disease affecting Ashkenazi Jews, with a frequency of approximately 1 in 850 individuals having Gaucher disease and 1 in 18 individuals being carriers. Once Gaucher disease is suspected based on clinical presentation, the first line of testing is measuring the glucocerebrosidase (GBA) enzyme activity.

The GBA enzymatic assay has been developed as a part of this study by modifying the conditions described by Urban et al (2008). The process of validation was divided into individual steps identified to provide the most optimum conditions for the implementation of this assay. This assay uses the fluorometric methodology to measure the level of enzymatic activity. The fluorometric signal is directly proportional to the concentration of GBA activity in the individual being assessed.

The evaluation of all components of the method validation included accuracy, precision, linearity, sample requirements, sample stability, and establishment of reference ranges. After the individual assessment of these components, it was concluded that the assay was successfully validated for the measurement of GBA activity, and it provides a valuable tool as the first line of testing for the diagnosis of Gaucher disease.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Irene De Biase, my committee chairman, for her guidance, assistance, and encouragement every step of the way. She has always motivated me throughout the whole process, allowing me to be a better scientist. I am very grateful for her guiding hands, ensuring that I was performing according to the guidelines, and for always going above and beyond what was needed of her. Thank you for your faith in me, for allowing me this wonderful chance to work with you and learning so much along the way. I am grateful to the other members of my committee, Dr. Marzia Pasquali and Dr. Nicola Longo, for also guiding me and giving valuable information and assistance during this process.

I am also grateful to all the members of the Biochemical Genetics Laboratory at Associated Regional and University Pathologists (ARUP), for their patience during the thesis process, and especially for always making me feel welcome and part of the group.

I am grateful to JoAnn Fenn, director of the Master's Program of Science in Laboratory Medicine and Biomedical Science, for her instruction, counsel, and encouraging support throughout the entire degree.

A personal thank you for my family, for my husband George E. Brown, and my sons Daniel and Nicholas Brown, for their patience and support throughout my Master's degree. Without their help and understanding, I would have never been able to finish my degree. Thank you to my mother Sandra Cisneiros, for her constant support and for coming

from Brazil to help me, so that I could be able to finish my thesis, and a special thank you to my father Fernando Cisneiros, for truly inspiring me throughout my life about the importance of pursuing my dreams, and for supporting me unconditionally in my academic decisions.

CHAPTER I

INTRODUCTION

Lysosomal storage disorders

Lysosomes are membrane-enclosed organelles that function as the digestive system of the cell by degrading intracellular materials. Lysosomal storage disorders (LSDs) are a heterogeneous group of defects affecting lysosome homeostasis. They are usually caused by the deficiency of a single hydrolase; however, they can be due to defects in lysosomal or nonlysosomal proteins essential to lysosomal biogenesis/function. In this group of disorders, more than 50 different diseases are recognized. These disorders are characterized by the accumulation of undigested or partially digested macromolecules, which affects cellular function and results in clinical abnormalities (*1, 2*). Taken individually most LSDs are rare; however, taken together, LSDs are relatively common with a cumulative incidence of approximately 1 in 5,000 live births (*3*). Moreover some disorders are found in increased frequency in particular populations (*4*).

Laboratory investigations are critical for the diagnosis of LSD. Early diagnosis is important because of the availability of treatments, including enzyme replacement therapy.

Gaucher disease

Gaucher disease (GD) is an autosomal recessive disorder caused by deficiency of the lysosomal hydrolase glucocerebrosidase (GBA) (EC 3.2.1.45), required for the degradation of glycosphingolipids. Glycosphingolipids are essential components of the cell plasma membrane, predominantly in the nervous system. Several lysosomal storage disorders are caused by a deficiency in one of the steps in the glycosphingolipids catabolic pathway. In Gaucher disease, as a result of GBA deficiency, the body is unable to breakdown glucocerebroside, thus allowing the accumulation of glucosylceramide and glucosphingosine within the lysosomal cells. This substrate accumulation prevents cells within the monocyte-macrophage system to function properly (*5, 6*).

The pathologic hallmark of GD is the presence of Gaucher cells in organs such as bone, bone marrow, liver, spleen, and lymph node parenchyma. The morphologic features of Gaucher cells are unique; these cells range from 20 to 100 μm in diameter and they can be identified by the presence of a small eccentric nuclei, and cytoplasm with crinkles and striation. The eccentric nuclei and the crinkles and striation found in Gaucher cells are caused by the abundance of lipids in the cell's cytoplasm, resulted from the enzyme absence in the organism (*5, 6*).

Clinical presentation and classification of Gaucher disease types

The clinical presentation of GD consists in a multi-system disorder, whose features include peripheral blood cytopenias, hepatosplenomegaly, bone disease, and, in some patients, neurological manifestations (*5, 7, 8*). Traditionally, the classification of GD into its three subtypes is dependent primarily on the presence or absence of neurological

features.

Type 1 Gaucher Disease (GD1) is the most common form, and accounts for almost 95% of GD cases among Caucasians. Its primary characteristic is the lack of central nervous system (CNS) involvement. The systemic features associated with this subtype include hepatosplenomegaly, bleeding, cytopenias, gammopathies, bone pain, avascular necrosis, and osteopenia. Disease development can begin any time between childhood and adulthood, and mild cases can remain asymptomatic. Because of widely used therapeutic treatment options, which include enzyme replacement therapy (ERT), substrate reduction therapy (SRT), orthopedic interventions, and biphosphonates for osteopenia, life expectancy is normal (*5, 7, 8*).

Type 2 Gaucher Disease (GD2) is the most severe form of the disease. Usually clinical symptoms appear before one year of age with severe neurologic involvement. GD2 patients present developmental delay, strabismus, organomegaly, and mild pancytopenia. Rare cases can present with congenital ichythosis and nonimmune hydrops fetalis. Life expectancy is < 2 years. Because of the severe nature of the clinical symptoms, the only treatment available is palliative to increase the level of comfort of the patients (*5, 7, 8*).

Type 3 Gaucher Disease (GD3) was formerly known as juvenile Gaucher disease, because the age of presentation ranges from childhood to adolescence. Its primary characteristic is a subacute involvement of the CNS, which is slowly progressive and it is characterized by developmental delay, strabismus, and cognitive regression in some patients. Systemic features are also present, and can include, in addition to the same symptoms found in GD1, growth retardation, cardiac valve calcification, and kyphosis. Treatment involves ERT for systemic features, with bone marrow transplantation being an

option for some patients. Most patients die in late childhood to mid adulthood (*5, 7, 8*).

Gaucher disease type 1 is the most common inherited genetic disease affecting Ashkenazi Jews (Eastern, Central, and Northern European Jewish ancestry). In this population, approximately 1 in 850 individuals have Gaucher disease and 1 in 18 individuals are carriers (*9*). The prevalence of GD in the general population is 1:50,000. The prevalence of neuropathic Gaucher disease (types 2 and 3) is low and varies among ethnic groups, with non-Europeans presenting higher frequency (*9*).

There are over 200 known mutations responsible for the decrease or complete loss of function of the GBA enzyme (*10*). Table 1 contains the most common mutations found in the *GBA* gene. Testing for these mutations would identify 90-95% of Gaucher disease cases in the Ashkenazi Jews, and 50-75% of cases in the general population.

Gaucher disease diagnostic testing

Once Gaucher disease is suspected based on clinical presentation, the first line of testing is measuring the glucocerebrosidase (GBA) enzyme activity (*1, 2*). The detection of very low levels of GBA can be used to diagnose patients with all subtypes of GD. The acceptable specimen types for the enzymatic test include dried blood spots, white blood cells isolated from whole blood, and cultured skin fibroblasts (*1, 2, 9*). The GBA enzymatic assay uses enzyme-specific artificial substrates, recognized and cleaved by the endogenous enzyme. Different substrates are available commercially that can be detected by fluorometric or mass spectrometry methods. In patients lacking GBA enzymatic activity, the artificial substrate cannot be cleaved, and no tag is released and measured. Further diagnostic confirmation is obtained by molecular testing. Molecular testing can be useful

to predict clinical outcome, since genotype-phenotype correlations have been described for some *GBA* mutations. Moreover, molecular testing is used to identify family members that are carriers for the disease in *at-risk* families.

Gaucher disease treatment options

Enzyme replacement therapy (ERT)

Enzyme replacement therapy works by administrating an artificially synthetized enzyme, which acts like the naturally occurring glucocerebrosidase enzyme, allowing the breakdown of accumulated glucosylceramide and glucosphingosine in the affected organs (*11*). Treatment needs to be administered throughout the patient's life span, because the absence of the enzyme is corrected permanently by ERT.

In 1991, alglucerase (Ceredase®; Genzyme, Boston, MA) was approved by the Food and Drug Administration (FDA) for ERT in GD patients (*12*). However, during manufacturing times, shortage of ERT drove the pharmaceutical market to expand the options available for GD patients. And now there are several medications available in the market worldwide for ERT (*11-15*).

Imiglucerase (Cerezyme®; Genzyme, Boston, MA) is a recombinant DNA-produced analogue of human glucocerebrosidade, made using Chinese hamster ovary cells, indicated for long-term enzyme replacement therapy for pediatric and adult patients with a confirmed diagnosis of Type 1 GD (*13*). Patients are recommended the use of this therapy once one or more of the following conditions are present: anemia, thrombocytopenia, bone disease, and hepatomegaly or splenomegaly (*13*).

Taliglucerase alfa (Elelyso™; Protalix Biotherapeutics, Carmiel, Israel) contains

some amino acid differences in its composition, when comparing to the original human glucocerebrosidase. It is produced by genetically modifying carrot cells (*14*).

Velaglucerase alfa (VPRIV®; Shire Human Genetic Therapies Inc.) was the third ERT approved for treatment. It is produced with human fibroblasts cell lines (*15*).

Patients with GD type 1 are the most suitable targets for ERT, because these medications are not capable to cross the brain-barrier, thus not being able to alter the disease course of patients that present with central nervous system involvement. One of the biggest challenges that physicians face when utilizing ERT is finding the right dosage that works with each individual (*11*). In these cases, the availability of biomarkers prove invaluable, because they allow physicians to monitor levels of glucocerebrosidase byproducts present in the system, and adjust therapy on an individual basis.

One important aspect influencing the efficacy of ERT is the age at which the treatment begins. Early ERT utilization is known to produce better results because it prevents long term complications (*4*).

Substrate reduction therapy (SRT)

In contrast with ERT, substrate reduction therapy's approach acts on the glycolipid accumulation found in patients with GD by reducing the substrate within the cells. One of the advantages in SRT is its ability to be given orally, in contrast with ERT which requires intravenous administration. Certain patients also have contraindications to the use of ERT as a primary form of therapy.

Miglustat (Zavesca®, Actelion Pharmaceuticals US) works by inhibiting the ceramide glucosyltransferase pathway, which is an important step in the biosynthesis of

glycolipids (*16, 17*). The therapeutic use of miglustat have been limited after a large group of patients utilizing this therapy experienced side effects that include diarrhea, weight loss, abdominal distension, and tremor, and it was noted that a reduction in symptoms caused by the substrate accumulation took a significant longer amount of time than with the utilization of ERT (*17*).

Eliglustat (Cerdelga®, Genzyme, Boston, MA) is a newer SRT option that is taken orally and is an inhibitor of the glucosylceramide synthase. It was found to have a good oral bioavailability, a high therapeutic index, and limited toxicity, especially when compared to Miglustat (*18, 19*). Patients can differ in their ability to metabolize eliglustat. Slower and poorer metabolizers retain the drug longer than patients that are ultra-rapid metabolizers, who need higher concentrations to achieve a similar therapeutic effect in the organism. Because of the different response to the drug, eliglustat requires specific laboratory testing to assess the patients' ability in metabolizing it (*20*).

Gaucher disease biomarkers

Biomarkers are measurable indicators of disease progression, and can be used to monitor therapeutic interventions over a period of time. A good predictor biomarker is an indicator whose levels are abnormal in the disease state and change upon treatment. A good biomarker is also specific for a particular condition. However, not all biomarkers are specific for one condition only. Some biomarkers are nonspecific but they can still be accurately used as a therapeutic tool, when combining with clinical findings and/or other biomarkers.

In the case of GD, the treatment options are very expensive. The use of biomarkers

to monitor effectiveness of treatment is fundamental not only medically but also financially. In most cases, the biomarkers used for therapeutic monitoring of GD will have a decrease in levels on the blood stream upon successful treatment. And if the reduction experienced upon treatment is not the level that was medically desired, physicians can use those results to adjust doses and try to reach the appropriate therapeutic level that the patient needs (*21*).

Plasma chitotriosidase

In healthy individuals, plasma chitotriosidase (CT) in expressed in tissue macrophages and secreted after activation. In GD disease patients, macrophages have abnormal lipid-filled cytoplasm and produce markedly elevated amounts of CT (*21-23*). Once treatment is administered, CT levels decrease according to the response from the patient. There is variability among patients in CT levels reached after therapy. However, lack of a significant decrease in CT levels may indicate the need for a revision of the initial therapeutic doses (*6*).

About 1 in every 20 patients does not show any CT activity in plasma. These GD patients require different biomarkers for therapeutic monitoring (*24*).

Angiotensin converting enzyme

Angiotensin converting enzyme (ACE) is a nonspecific indicator of lipid storage, and elevated levels reflect the accumulation of lipids in the cytoplasm of Gaucher cells during disease progression (*25*).

ACE is nonspecific as biomarker, and can be found in elevated levels in other

disorders. However, because ACE levels decrease significantly in GD patients after starting treatment, the levels of ACE can be used for therapeutic monitoring in addition to other biomarkers. Because of its nonspecific nature, laboratory findings of ACE should be correlated with clinical finding (*21*).

Tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAP) is an expressed marker for macrophages, and it is known to predict bone disease when presented in elevated levels. It consists of two isoforms, type 5a and 5b, both having different biochemical, clinical, and biological properties (*26*).

TRAP is also a nonspecific biomarker for Gaucher disease (*6*). It will present itself in high levels in untreated individuals, and decrease its value when treatment is successful.

Pulmonary-and-activation-regulated chemokine

Pulmonary-and activation-regulated chemokine (PARC-CCL18) is expressed predominantly by macrophages. As a biomarker for GD, PARC concentrations have been shown to be elevated in the serum of untreated patients, and can also be measured in urine because of its excretion (*6, 21, 24*). However, PARC is not specific only for GD patients, and have been elevated in the presence of other diseases as well (*24*).

In contrast with chitotriosidase, whose presence cannot be detected in a percentage of the population, PARC can always be detected. And another advantage is the feasibility of measurement since enzyme-linked immunosorbent assay (ELISA) can be used to measure serum PARC levels (*21*).

Glucosylsphingosine

Glucosylsphingosine is a relatively new biomarker for Gaucher disease. Its usefulness is still undergoing investigation. Studies suggest that high levels of glucosylsphingosine will accumulate in brain tissues of Gaucher patients, especially in patients of type 2 and type 3 GD, where there is CNS involvement (*27, 28*).

The advantage of this biomarker, comparing to previously used ones, is the ability to predict disease development in patients where there is neurological involvement. It is also proposed that this biomarker, present in high levels, may contribute to disease development in the bones. It will interfere with normal osteoblast function, and therefore causes low bone mineral density, which in turns affects bone health (*28*).

Table 1. Common mutations in the *GBA* gene that cause Gaucher disease (*10*).

cDNA/ Base Sub.	Amino Acid #	Genome #	AA Sub.	Phenotypic Effect	Frequency	Enzymatic Effect	
						Catalytic	Stability
c.84G>GG	NA	1035	NA	Severe	Very Common	Null	Null
NA G>A	IVS2+1	1067	NA	Severe	Common	Null	Null
c.1226A>G	370	5841	Asn → Ser	Mild	Very Common	↓ Activity	Stable
c.1297G>T	394	5912	Val → Leu	Severe	Common	↓ Activity	Stable
c.1342G>C	409	5957	Asp → His	Severe	Common	↓↓↓ Activity	Unstable
c.1448T>C	444	6433	Leu→ Pro	Severe	Very Common	↓↓ Activity	Unstable
c.1504C>T	463	6489	Arg → Cys	Mild	Common	↓ Activity	Stable
c.1604G>A	496	6883	Arg → His	Very Mild	Very Common	↓ Activity	Stable

CHAPTER II

MATERIAL AND METHODS

Chemicals and reagents

Protein quantification was performed using the BioRad protein assay kit II (BioRad Laboratories, Hercules, CA; Cat #500-0002) following the SOP in use in the biochemical genetic laboratory at ARUP (BCG-03-3415, Cell Lysate Preparation and Protein Concentration Determination by UV Spectrophotometry).

Citric acid monohydrate granular (C6H8O7:H2O; Cat# JT0119-1) and glycine (CH2(NH2)COOH; Cat #97061-128) were obtained from VWR Scientific (Radnor, PA). Sodium carbonate, anhydrous (Na_2CO_3; Cat #3605-01) was obtained from JT Baker (Center Valley, PA), and Methylumbelliferone Sodium Salt (Cat #152475) from MP Biomedical (Santa Ana, CA). Phosphoric acid (Cat# 452289-50mL), Sodium phosphate, dibasic (Na2HPO4; ARUP Cat# 44716), Tween-20 (Cat#P2287-100ML) and Sodium taurocholate (Cat# T4009-250MG) were obtained from Sigma–Aldrich (St. Luis, MO).

4-methylumbelliferyl-beta-D-glucopyranoside was obtained from Marker Gene Technologies (Eugene, OR; Cat#M1940) or Sigma-Aldrich (St. Luis, MO; Cat# M9766). Detailed description of reagent preparation is in the standard operating procedure (SOP) (see Appendix 1).

Isolation of leukocytes

To measure GBA activity, white blood cells (leukocytes) were isolated from whole blood collected in three different collection tubes: Acid Citrate Dextrose (ACD; yellow top), ethylenediamine tetraacetic acid (EDTA; purple top), and sodium heparin (green top) tubes. Leukocytes were isolated from whole blood using a procedure validated in the biochemical genetic laboratory at ARUP (BCG-03-3416, Leukocyte Isolation from Whole Blood). Briefly, leukocytes are isolated by centrifugation after specific lysis of red blood cells with a buffer containing ammonium chloride [155 mmol/L], sodium bicarbonate [10 mmol/L], and EDTA [0.1 mmol/L]. After centrifugation, the lysed red blood cells are discarded with the supernatant. This step is performed twice to minimize red blood cell contamination. After a final wash in saline, the leukocytes pellets were stored at ≤ - 65℃ prior to the utilization.

Sample preparation and protein determination

Leukocytes pellets were resuspended with chilled nanopure water, and cell lysis was performed by sonication (three 10 s 8-watts bursts) utilizing the Fisher Scientific Sonic Dismembrator Model 100. This procedure has been validated in the biochemical genetic laboratory at ARUP (BCG-03-3415, Cell Lysate Preparation and Protein Concentration Determination by UV Spectrophotometry). Samples are kept on ice during the entire process. The sonicator probe tip is washed in water between samples to avoid any sample contamination between different specimens.

Protein quantification was performed for each sonicate. This value is used when calculating the GBA activity, which is expressed in nanomoles of substrate hydrolyzed per

hour per milligram of protein. An aliquot of the leukocyte lysate is used to quantify the protein concentration using the Bio-Rad Protein Assay, based on the method of Bradford. This procedure involves the addition of an acidic dye to protein solution, and subsequent measurement of the absorbance. Comparison to a Bovine Serum Albumin (BSA) standard curve provides a relative measurement of protein concentration. The Bio-Rad Protein Assay was performed using the microplate assay (using the Safire2™ plate reader), read at a wavelength of 587 nm (according to BCG-03-3415). Six calibrators were included in the BSA standard curve (0 – 0.59 mg/mL).

Glucocerebrosidase assay in leukocytes

The GBA enzymatic assay uses the synthetic fluorogenic substrate 4-methylumbelliferyl-beta-D-glucopyranoside. The assay has been developed modifying the conditions described by Urban et al. (2008). The SOP validated during this project is detailed in the Appendix. Briefly, two duplicate reactions were prepared for each control/patient by combining 20 μL of cell lysate and 80μL of 4-methylumbelliferyl substrate in buffer (50 mM citric acid, 176 mM sodium phosphate, 0.01% Tween-20 and 20 mM sodium taurocholate; pH 5.4) in a 96-wells plate. In each run, a duplicate blank was included. Samples were incubated at 37˚C for 2 h using the BioRad DNAEngine® Peltier Thermal Cycler (PCR). At the end of the 2 h incubation, the enzyme reaction was stopped by adding glycine carbonate buffer (pH 10.0). After stopping the enzyme reaction, an aliquot of the reaction mixture was transferred from the PCR tube strips to a plate, and further diluted by adding more glycine carbonate buffer. Fluorescence was read using a Safire2™ 4-monochromator scanning fluorescence plate reader (Tecan, Männedorf,

Switzerland).

The instrument setting was:

- Measurement mode: Fluorescence Top (number of reads: 10)
- Gain (Manual): 49
- Excitation (EX) 365nm
- Emission (EM) 441nm
- Excitation bandwidth: 10.0 nm
- Emission bandwidth: 10.0 nm

Calculations

GBA activity was calculated for each sample measuring the reaction product (4-methylumbelliferone) against a standard calibration curve. The calibration curve is established with each run. The average fluorescence reading of the duplicate reactions is used in the calculations, and it must be within 20% coefficient of variation (CV%). After subtracting the average fluorescence of the duplicate blanks, the average fluorescence reading is divided by the slope of the standard curve, and then multiplied by several factors that account for sample dilution, incubation time, and milligram of proteins used.

Gaucher disease patients have low to almost undetectable GBA activity. Carriers of *GBA* mutations may display lower activity; however, considerable overlap exists with the normal range. Therefore, this test is not helpful in identifying GD carriers.

The GBA activity is expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein.

Calculation:

$$\text{GBA activity} = \left[\frac{(\text{fluor. of activity} - \text{fluor. of blank})}{(y)} * 0.24\right] * 6.25 \div 2 \div (\text{mg of protein used for assay})$$

Note: The above calculations are based upon:

* 0.24 = correction to account for final concentration in samples versus calibrators

* 6.25 = correction for final volume

/ 2 = correction of 120 min to 60 min

mg of protein used for assay = 0.020*protein conc. of sonicate (mg/mL)

y = slope of the standard calibration curve

Characteristics of known Gaucher disease patients used in the clinical sensitivity/specificity study

For this study, whole blood samples were collected from a cohort of five Gaucher disease patients. All five patients are followed at the Metabolic Clinic at the University of Utah, under the supervision of Dr. Nicola Longo, Chief of the Division of Medical Genetics, Professor of Pediatrics, and Adjunct Professor of Pathology at the University of Utah. For the sample collection, an informed consent was obtained by all patients. Demographic, clinical, and laboratory data were obtained by retrospective chart review. This study was reviewed and approved by the Institutional Review Board at the University of Utah. Table 2 summarizes the clinical features of the five GD patients.

All five patients were diagnosed after clinical presentation; the average age of

diagnosis was 15.8±7.7 years. In all cases, the diagnosis was confirmed by DNA sequencing of the *GBA* gene (Table 3). All patients are currently on enzyme replacement treatment (biweekly), and have been for several years. For this study, we did not have access to ERT-naïve patients.

Table 2. Gaucher disease patients' clinical features.

	Gender	Age (years)			Presenting Symptoms	Therapy	Outcome
		Onset	DX	Current			
A	M	8	12	37	Hepatosplenomegaly and bone lesions	ERT (biweekly)	Normal development; multiple orthopedic problems; reduced mobility; severe pain
B	F	13	14	61	Severe splenomegaly	ERT (biweekly)	Normal development; splenomegaly; unrestricted mobility; mild pain
C	M	UNK	9	45	Hepatosplenomegaly and bone pain	ERT (biweekly)	Normal development; unrestricted mobility; severe pain
D	F	UNK	15	34	Bone pain and hip necrosis	ERT (biweekly)	Normal development; unrestricted mobility; mild pain
E	M	UNK	29	45	Bone disease (mild)	ERT (biweekly)	Normal development; unrestricted mobility; mild pain

UNK = unknown

Table 3. Molecular characterization of Gaucher disease patients.

	GBA gene mutations	
Patient	Allele 1	Allele 2
A	c.1226A>G (p.N370S)	IVS2+1G>A[a]
B	c.1226A>G (p.N370S)	c.1448T>C (p.L444P)
C	c.1226A>G (p.N370S)	c.644C>A (p.A215D)
D	c.1226A>G (p.N370S)	IVS8+1G>T[b]
E	c.1226A>G (p.N370S)	c.1226A>G (p.N370S)

[a] Pathogenic mutation (impaired splicing). [b] Unpublished mutation, likely pathogenic (impaired splicing).

CHAPTER III

RESULTS

Test development

The main aim for this project was to develop and validate an enzymatic assay to measure the activity of the lysosomal enzyme glucocerebrosidase (or acid β-glucosidase, EC 3.2.1.45) in leukocytes for the diagnosis of Gaucher disease. To develop the assay and validate it for the use in the biochemical genetic laboratory at ARUP, the conditions described in Urban et al. (2008) were used with minor modifications.

Effects of commercial substrate used

This enzymatic assay uses the synthetic fluorogenic substrate 4-methylumbelliferyl-beta-D-glucopyranoside. The assay combines leukocyte lysate with the substrate prepared in buffer. We tested the 4-methylumbelliferyl-beta-D-glucopyranoside produced by two companies: Marker Gene Technologies (Eugene, OR; Cat#M1940) and Sigma-Aldrich (St. Luis, MO; Cat# M9766). Four leukocyte lysates were tested using the two different substrates, three obtained from healthy controls and one from a GD patient (patient E; see Table 2). All patients were run in duplicate. A duplicate blank was run with each substrate. Table 4 shows the average fluorescence detected in the blank and in the lysates, and the corresponding GBA activity.

The background noise, due to the non-enzymatic breakage of 4-methylumbelliferyl-beta-D-glucopyranoside, was present in the blank assayed with both substrates; however, the background was much higher using the Marker Gene Technologies (company 1) substrate. There was a difference in GBA activity between controls and the GD patient, with the GD patient presenting a markedly decreased activity compared to the controls. However, with the Marker Gene Technologies (company 1) substrate the GBA activity in the GD patient was 9% of the controls average while it was 4% using the Sigma-Aldrich substrate (company 2). Because of the modest background and the better discrimination between controls and patient, we elected to use the Sigma-Aldrich substrate for the assay validation.

Effects of sodium taurocholate concentration

GBA requires interaction with negatively charged lipids and the cofactor saposin C for optimal activity. Detergents (like Tween-20) and negatively charged bile salts (such as sodium taurocholate, TA) allow for optimal GBA activity at acidic pH (buffer pH = 5.4) in vitro. We tested two sodium taurocholate concentrations: 10mM and 20mM. Four leukocyte lysates were tested using the two different buffers, three obtained from healthy controls and one from a GD patient (patient E; see Table 2). All patients were run in duplicate.

Table 5 shows the average fluorescence detected in the blank and in the lysates, and the corresponding GBA activity using the Sigma-Aldrich substrate. The background noise in the blank was similar with either concentration. GBA activity in the GD patient was higher using 10mM sodium taurocholate, possibly because of increased specificity using

higher sodium taurocholate concentration; although the experiment was not repeated. We elected to use bGLU-PC buffer with 20mM sodium taurocholate for the assay validation.

Calibration curve

The calibration curve is prepared shortly before reading the plate by diluting the standard working solution in Glycine Carbonate Buffer. An example of the calibration curve built using the average readings among 8 different standard curves (mean ±SD) is presented in Figure 1. The theoretical concentrations: 0, 0.12, 0.52, 1.03, 2.0 and 2.53nmol/mL are on the x-axis, while the respective readings by the instrument at the fixed wavelength (365.0/441.0nm) are on the y-axis (RFU = relative fluorescence units). The curve is forced through the zero. The regression line is determined by excel via least squares method (1st order). The slope of the regression is used in the calculations of the GBA activity; inter-assay variability of the slope within the 8 experiments had a coefficient of variation (CV%) <15%.

Specimen type

The effect on GBA activity of the anticoagulant used in the blood collection tubes was evaluated by testing three different collection tubes. Blood from three healthy controls was collected in Acid Citrate Dextrose (ACD; yellow top), ethylenediamine tetraacetic acid (EDTA; purple top), and sodium heparin (green top) tubes, and processed at the same time.

The anticoagulant used in the blood collection tubes seems to affect GBA activity. Samples collected using heparin tubes display lower activity, markedly reduced in two controls (Table 6). Samples collected in EDTA tubes displayed very similar activity

compared to the ACD tubes in two controls, and mildly reduced in one (Table 6). Based on these results, ACD tubes and EDTA tubes are both accepted as blood collection tubes. Validation experiments were performed using both ACD tubes and EDTA tubes, depending on sample availability. The normal ranges include results obtained with both sample types.

Specimen stability

Stability of the whole blood prior to the white blood cells isolation was evaluated by storing blood samples at room temperature up to 3 days from collection. Leukocytes were isolated at time 0, 2, and 3 days, and GBA activity was evaluated. Whole blood was collected from two healthy controls (a 25-year old male [25M] and a 37-year old female [37F]), in ACD tubes. The T0 point was immediately processed, while the other two tubes were processed at 2 and 3 days post blood draw, after being stored at room temperature. All cell pellets were stored at -65℃ prior to sample analysis. GBA activity was evaluated in all samples in the same experiment, using lysates prepared that day from the pellets. We observed a decrease in enzymatic activity after two days, but GBA activity did not change between day 2 and day 3 (Table 7). We concluded that samples received in the lab were acceptable up to 3 days from collection. However, further experiments are needed to evaluate stability after 3 days, as well as to verify the dramatic drop in GBA activity between the T0 and T2 more samples need to be analyzed.

To assess the leukocyte lysates stability once the pellet has been sonicated and upon storage at -65°C, we evaluated GBA activity in one normal sample that was sonicated, and aliquoted in frozen lysates kept at -65°C prior to the analysis. This was used as additional

control for each of the validation runs. We had a total of 10 values from 8 independent experiments over an eight-month period. The mean ±SD activity was 6.7±1.3 nmols/h/mg protein, the CV% was 19%, which is in line with the inter-assay imprecision observed (see assay validation).

Method validation

Accuracy

To assess the accuracy of the assay, GBA activity was measured in three proficiency testing specimens obtained from ERNDIM (European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism), as part of the lysosomal enzymes scheme. Samples were tested after at least a freeze/thaw cycle, after they were used for proficiency testing for other assays currently in use in the biochemical genetic laboratory at ARUP.

These proficiency samples were lyophilized fibroblasts, which were reconstituted in water prior to the analysis and quantified. The results were compared to the other participant laboratories (Table 8). All samples displayed normal GBA activity. The CV% between our result and the average result of all participating labs was less than 10% for all samples.

Additionally, to test the effect of lower protein concentration, we measured the accuracy of the assay using different dilutions of the same sample. GBA activity was measured in two normal leukocyte samples (collected in ACD tubes; from a 43- and a 55-year old female) in triplicate at three different protein concentrations (1X, 2X, and 10X dilutions), and in three different experiments. All reactions were run in duplicate.

Table 9 and 10 show the intra- and inter-assay variability at three different protein concentrations. GBA activity was linear within a broad range of protein concentrations: 1.82 - 0.11 mg/mL.

The intra-assay variation was ≤ 20% at all concentrations (2.8 - 11.6%) with a recovery of 100%±10%. The inter-assay variability was ≤ 20% at all concentrations for leukocyte 2, but leukocyte 1's variability was 23% at the lowest concentration (0.18mg/mL). However, recovery was 100%±10% at all concentrations.

Precision

The intra- and inter-assay variation in GBA activity was evaluated in leukocyte samples obtained from a normal control (from a 26-year old female) and from a Gaucher disease patient (patient A; see Table 2). Samples were isolated from whole blood samples collected in EDTA tubes (purple top), and stored at -65°C prior to the analysis. Each sample was assessed three times within a sample run, and in three different experiments (Table 11A and B). All reactions were run in duplicate.

The intra- and inter-assay variation in GBA activity was ≤ 20% in the normal control (Table 11A). The fluorescence detected in the GD patient was low (52 – 80 RFU), and within the blank average 62.7±22.9 (see interference section below). The variability was much higher in the GD patient sample where GBA activity is markedly reduced, although protein concentration was similar (Table 11B).

Reference range

To define the normal reference range for GBA activity in leukocytes, we evaluated enzyme activity in 32 normal samples. All leukocyte samples used to determine the normal range were extracted from whole blood samples collected in ACD or EDTA tubes, and processed according to the SOP. Cell pellets were stored at -65°C prior to the analysis. 16 samples were collected from healthy donors, and 16 samples were clinical samples sent to the biochemical genetic laboratory for unrelated testing, and de-identified according to ARUP procedures. Carrier status for Gaucher disease cannot be excluded in this cohort, since molecular testing was not performed. The mean activity was 11.94 nmol/h/mg protein (± 4.48 standard deviation).

The reference interval was established utilizing the software EP Evaluator (Release 10; Data Innovations) using the parametric analysis (Figure 2) with a confidence ratio > 0.26. Figure 2 showed the distribution of the GBA activity normal values (*N = 32*). Based on this distribution, the normal range is 3.7 – 21 nmol/hr/mg protein.

Interferences

In each experiment, a blank was run in duplicate to determine the background fluorescence in the assay buffer. The duplicate blanks contain water, and are incubated in the assay buffer together with controls and samples. All reactions are stopped by glycine carbonate buffer. The calibrator 1 in the standard calibration curve assesses the background fluorescence in the stopping buffer. Neither displays a significant fluorescence. Over approximately eight months, including all experiments run for this validation, the mean fluorescence ± SD of the blank was 62.7±22.9 (*N = 8*), CV% = 36.6%, less than 0.5% the average value for the highest calibrator (2.53 nmol). The mean fluorescence ± SD of

calibrator 1 (0 nmol) was 11.6±6.9 (*N = 8*), CV% = 59.3%, less than 0.1% the average value for the highest calibrator (2.53 nmol).

Clinical significance

All samples collected from healthy donors or sent to our clinical lab for unrelated testing displayed GBA activity. Although carrier status for Gaucher disease cannot be excluded in this population since molecular testing was not performed.

We had access to leukocytes from five Gaucher disease patients on biweekly enzyme replacement therapy. GBA activity was markedly reduced in all five samples, and outside the normal range (Table 12). All these patients have been on ERT for several years; unfortunately, we did not have access to ERT-naive patients to test our assay.

Table 4. Average fluorescence and GBA activity in the blank, a GD patient (patient E; see Table 2) and three healthy controls using 4-methylumbelliferyl-beta-D-glucopyranoside produced by Marker Gene Technologies (company 1) or Sigma-Aldrich (company 2).

	Fluorescence (RFU)		**GBA Activity (nmol/h/mg)**	
	Company 1	**Company 2**	**Company 1**	**Company 2**
Blank	701.5	72.0	0	0
GD patient	761.0	112.5	1.2	0.5
Control 1	1361.5	1503.0	11.8	18.1
Control 2	1787.0	1489.0	12.9	12.4
Control 3	2209.0	1008.0	15.5	7.1

Units: fluorescence expressed in relative fluorescence units; GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein.

Table 5. Average fluorescence and GBA activity in the blank, a GD patient (patient E) and three healthy controls using bGLU-PC buffer with 20mM or 10mM sodium taurocholate.

	Fluorescence (RFU)		GBA Activity (nmol/h/mg)	
	20mM TA	**10mM TA**	**20mM TA**	**10mM TA**
Blank	72.0	77.0	0.0	0.0
GD patient	112.5	191.0	0.5	1.6
Control 1	1503.0	1464.5	18.1	17.6
Control 2	1489.0	1424.0	12.4	11.8
Control 3	1008.0	959.5	7.1	6.7

Units: fluorescence expressed in relative fluorescence units; GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 6. Variability in GBA activity due to sample type.

	GBA Activity (nmol/h/mg)			
	ACD	**EDTA**	**Heparin**	**CV %**
Control 1	13.4	12.9	12.8	2.3%
Control 2	11.4	11.6	6.0	33.1%
Control 3	13.7	7.5	6.8	41.0%

Units: GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 7. Variability in GBA activity due to sample stability. The cumulative CV% of the results obtained with the three collection tubes is indicated.

	GBA Activity (nmol/h/mg)			
	T0	**T2**	**T3**	**CV %**
25M	12.0	4.9	5.3	53.9%
37F	7.4	4.7	4.3	30.8%

Units: GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 8. Glucocerebrosidase activity in three proficiency samples. Results were compared to the mean of participant labs. N indicated the number of participants.

ERNDIM sample	N	GBA Activity (nmol/h/mg) All labs	Your lab	CV %
2015.01	58	248.0	241.7	1.8%
2015.03	61	143.0	154.5	5.5%
2015.04	62	336.0	374.7	7.7%

UNITS: nanomoles of substrate hydrolyzed per hour per milligram of protein.

Table 9. Effect of low protein concentrations on the GBA activity (Normal leukocyte 1)

Normal Leukocytes 1

Proteins [mg/mL]		Intra-assay Fluor	Intra-assay GBA		Inter-assay Fluor	Inter-assay GBA	
		Mean			Mean		
	1	2430	7.9		2430	7.9	
1.84	2	2220	7.2		2181	10.3	
	3	2207	7.2		2838	9.3	
Average			7.4			9.2	
CV%			5.6%	Recovery		13.0%	Recovery
	1	1112	3.5	100.8%	1112	3.5	92.5%
0.92	2	1168	3.7		1110	5.1	
	3	1235	4.0		1281	4.0	
Average			3.7			4.2	
CV%			5.4%	Recovery		19.0%	Recovery
	1	251	0.7	107.9%	251	0.7	103.0%
0.18	2	290	0.8		275	1.1	
	3	306	0.9		386	1.0	
Average			0.8			0.9	
CV%			11.6%			23.0%	

Units: fluorescence expressed in relative fluorescence units; GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 10. Effect of low protein concentrations on the GBA activity (Normal leukocyte 2)

Normal Leukocytes 2

		Intra-assay			**Inter-assay**		
Proteins [mg/mL]		**Fluor** Mean	**GBA**		**Fluor** Mean	**GBA**	
	1	2035	10.4		2035	10.4	
1.14	2	1882	9.6		2113	15.6	
	3	1897	9.6		2555	13.0	
Average			9.9			13.0	
CV%			4.4%	Recovery		20.0%	Recovery
	1	1014	5.1	103.1%	1014	5.1	96.8%
0.57	2	1048	5.2		1011	7.3	
	3	993	4.9		1321	6.5	
Average			5.1			6.3	
CV%			2.8%	Recovery		18.0%	Recovery
	1	235	1.0	105.0%	235	1.0	99.0%
0.11	2	236	1.0		217	1.3	
	3	248	1.1		376	1.5	
Average			1.0			1.3	
CV%			3.6%			20.0%	

Units: fluorescence expressed in relative fluorescence units; GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 11. Intra- and inter-assay variation in leukocytes from a normal control (A) and from a GD patient (patient A) (B)

A **Normal Leukocytes**

Proteins [mg/mL]		Intra-assay Fluor	Intra-assay GBA	Inter-assay Fluor	Inter-assay GBA
		Mean		Mean	
0.63	1	1369	11.8	1369	11.8
	2	1409	12.2	1631	12.9
	3	1413	12.2	1130	11.6
Average			12.1		12.1
CV%			1.8%		5.8%

B **GD patient Leukocytes**

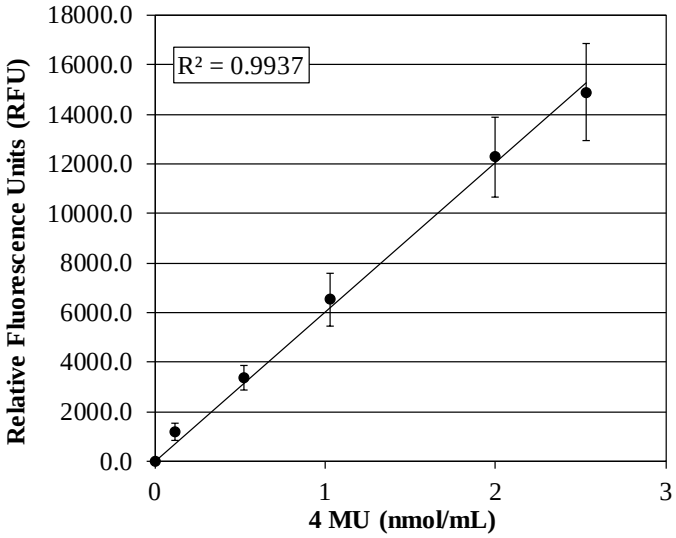
Proteins [mg/mL]		Intra-assay Fluor	Intra-assay GBA	Inter-assay Fluor	Inter-assay GBA
		Mean		Mean	
0.46	1	70	0.1	70	0.1
	2	79	0.2	80	0.5
	3	74	0.1	52	0.2
Average			0.1		0.2
CV%			43.5%		90.8%

Units: fluorescence expressed in relative fluorescence units; GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein

Table 12. GBA activity in five GD patients. Normal range: 3.7 – 21 nmol/h/mg protein

	Gender	Age (years)	Collection tube	Storage (days at RT)	GBA activity
A	M	37	EDTA	1	0.1
B	F	61	EDTA	2	1.4
C	M	45	EDTA	1	0.7
D	F	34	EDTA	1	0.2
E	M	45	EDTA	2	0.5

Units: GBA activity expressed in nanomoles of substrate hydrolyzed per hour per milligram of protein



Calibrators	Mean	SD	CV%
0	11.6	6.9	59.3%
0.12	1200.4	361.2	30.1%
0.52	3383.8	485.0	14.3%
1.03	6537.7	1054.1	16.1%
2	12282.1	1629.7	13.3%
2.53	14887.4	1961.9	13.2%

R^2	0.9937	0.0	0.5%
R	0.9969	0.0	0.2%

Blanks	62.7	22.9	36.6%
Slope	6032.9	784.5	13.0%

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Figure 1. Standard calibration curve. The coefficient of variation (CV%) is indicated for each calibrator, including the zero, the blank, the slope and the R^2.

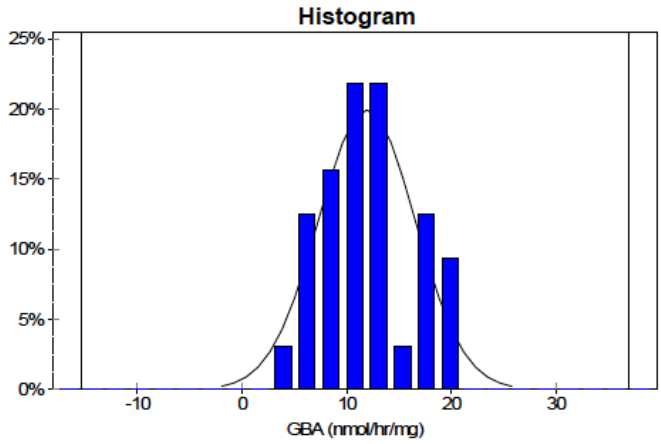

Figure 2. GBA activity distribution in normal leukocytes controls.

CHAPTER IV

DISCUSSION

Gaucher disease (GD) is an autosomal recessive disorder caused by deficiency of the lysosomal hydrolase glucocerebrosidase (GBA) (EC 3.2.1.45), required for the degradation of glycosphingolipids. GD is a multisystem disorder, whose features include peripheral blood cytopenias, hepatosplenomegaly, bone disease, and, in some patients, neurological manifestations (*5*, *7*, *8*). Patients with the less severe form of the disease (GD type I) benefit greatly from therapeutic intervention, which include enzyme replacement therapy. Because of improved clinical outcome after treatment, diagnosing GD patients is critical. The detection of very low levels of GBA enzyme activity can be used to diagnose GD. In this study, we aimed to develop and validate an enzymatic assay for use in the Biochemical Genetics Laboratory, which is part of ARUP Laboratories.

The GBA enzymatic assay has been developed modifying the conditions described by Urban et al. (2008). During test development, the optimum conditions for the assay were established. Chemical nonenzymatic breakage of the 4MU substrates can be a problem for enzymatic assays fluorescence-based because of high background in the blank. The commercial substrate obtained from Sigma-Aldridge displayed less background and was chosen for the assay validation. Moreover, we evaluated the effects of the anticoagulant used in the collection tubes by testing the three most commonly used collection tubes. We

tested Acid Citrate Dextrose (ACD; yellow top), ethylenediamine tetraacetic acid (EDTA; purple top), and sodium heparin (green top) tubes. In three normal controls, GBA activity was similar in ACD tubes and EDTA tubes and these sample types were considered acceptable as blood collection tubes. Reference ranges were established using samples collected in ACD and EDTA tubes. Because of the difference in GBA activity obtained using heparin tubes, further investigation is necessary to define a normal range to use for this sample type. Differences in enzymatic activity among different collection tubes have been previously observed in the biochemical lab during several validations for lysosomal enzymatic assays. Moreover, acceptability criteria that include or exclude sample types are usually published by laboratory to aid the users' test submission.

During method validation, we assessed accuracy, precision, and linearity of the assay. The method was adequately reproducible, with good intra and interprecision within the linearity range (1.82 - 0.11 mg/mL), and good accuracy. One important limitation of our study was the fact that we only had access to three proficiency-testing samples. The ERNDIM lysosomal enzymes scheme only provides eight samples each year for testing, and our use was further limited by the clinical lab need for these samples.

The clinical sensitivity and specificity of the assay was evaluated by testing normal controls and five GD patients. All samples collected from healthy donors or sent to our clinical lab for unrelated testing display GBA activity in the range previously reported (*9*). In patients lacking GBA enzymatic activity, the artificial substrate (4-MUF) cannot be cleaved, and thus no fluorescence is released. Both the fluorescence detected and GBA activity calculated were low in all five GD patients tested, and significantly different than in normal controls ($p < 0.0001$). The method differentiates clearly between normal activity

and GBA deficient patients. Because of the significant decrease in GBA activity, enzymatic activity measurements in GD patients may fall below the linearity range of the assay. Acceptability criteria for precision in these cases would be mean ± 0.5 nmol/h/mg protein. Table 11B clearly shows that precision can be affected when testing GD leukocytes, if activity is outside linearity: < 1 nmol/h/mg protein (normal range: 3.7 – 21). In these patients, activity will be reported as less than 1 nmol/h/mg protein, and considered consistent with Gaucher disease.

In conclusion, the enzymatic assay to test for GBA deficiency was successfully validated and can be used for the measurement of GBA activity both in GD patients as well as normal patients.

APPENDIX A

BETA-GLUCOSIDASE ACTIVITY IN LEUKOCYTES STANDARD

OPERATING PROCEDURE

Beta-glucosidase Activity, Leukocytes

Purpose

This assay is designed to measure the activity of the lysosomal enzyme β-glucosidase (also known as acid β-glucocerebrosidase, GBA; EC 3.2.1.45) in leukocytes to aid the diagnosis of Gaucher disease.

Principle

The lysosomal enzyme β-glucosidase catalyzes the breakdown of the glycolipid glucosylceramide (GlcCer) to ceramide and glucose. The deficiency of this enzyme causes Gaucher disease (OMIM 608013). The pathologic hallmark of GD is the presence of Gaucher cells in organs such as bone, bone marrow, liver, spleen, and lymph node parenchyma. The clinical presentation of GD consists in the presence of a multi-system disorder, whose features include peripheral blood cytopenias, hepatosplenomegaly, bone disease, and, in some patients, neurological manifestations. Traditionally, the classification of GD into its three sub-types (type I, type II and type III) is dependent primarily on the presence or absence of neurological features. Type 1 GD (GD1) is the most common form, and accounts for almost 95% of GD cases among Caucasians. Its primary characteristic is the lack of central nervous system (CNS) involvement.

This enzymatic assay uses the synthetic fluorogenic substrate 4-methylumbelliferyl-beta-D-glucopyranoside. The assay combines leukocyte lysate with the 4-methylumbelliferyl substrate prepared in buffer (50 mM citric acid, 176 mM sodium phosphate, 0.01% Tween-20 and 10 mM sodium taurocholate; pH 5.4). Samples are incubated at 37°C for 2 hours. The enzyme reaction is stopped by adding glycine carbonate buffer to the tubes and the fluorescence is measured against a

4-methylumbelliferone standard curve. In the absence of the enzyme, the artificial substrate cannot be cleaved and 4MU is not released.

Scope

This procedure is performed by a Technologist trainee, Technologist I, II or Medical Technologist in the Biochemical Genetics Laboratory. Results and run data, including all QC and other test parameters, are reviewed and approved (validated) by a Medical Technologist or Technologist II with 6 years of experience, or by a Medical Director. In urgent situations, a less experienced individual may validate the run, provided that an individual with appropriate experience also reviews and validates the data on the next shift that such a person is available. Once the run is validated, patient results may be verified in the LIS (released to clients) by the Technical Supervisor, Lead, or any Technologist that is trained and competent at performing the test.

Related Documents

CORP-PROC-7108, pH Meter Use and Maintenance
Safire2™ Plate Reader User Manual (version 1.5; 2005-12; Tecan)
Safire2™ Plate Reader Use and Maintenance
BioRad DNAEngine® Peltier Thermal Cycler (PCR) Use and Maintenance
BCG-03-3416, Leukocyte Isolation from Whole Blood
BCG-03-3415, Cell Lysate Preparation and Protein Concentration Determination by UV Spectrophotometry
CORP-PROC-7108, pH Meter Use and Maintenance
CORP-PROC-7073, Specimen Handling: Ordering and Viewing Except Tests

Personal Protective Equipment (PPE)

Performance of this procedure will expose testing personnel to potentially bio-hazardous material and possible chemical hazards. All specimens must be handled as potentially infectious material as outlined in the ARUP safety manual.
The reagent(s) and/or chemical(s) that are used in this assay may be hazardous to your health if handled incorrectly. A brief listing of precautions for each chemical hazard is included in the reagent section of this procedure.

More extensive information concerning the safe handling of the reagents and/or chemicals used in this assay, as well as other important safety information, may be obtained by consulting the Safety Data Sheet (SDS) and HAZ-COM manual, and ARUP safety manual. Before performing any part of this assay, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose the user to:
☒ Bloodborne pathogens
☒ Hazardous reagents

To perform this procedure the user must wear:
☒ Gloves
☒ Safety glasses, Face shield or equivalent
☒ Fully buttoned laboratory coat

To perform this procedure the user must use:
☒ Fume hood
☒ Disinfectant following procedure:
  ☒ Diluted Bleach (10% solution)
  ☒ Commercial disinfectant

Specimen

1. Specimen: Whole blood

2. Specimen Collection:
   Collect 3-5mL of whole blood in glass blood collection tube (1 mL is the minimum volume necessary to attempt the isolation of white blood cells). However, physiological variation in white cell count will influence the minimum amount of sample necessary for the extraction.
   - Acid Citrate Dextrose (ACD, Solution A or B) (yellow top) tubes are preferred; EDTA (purple top) tubes are accepted.

3. Specimen storage and transportation:
   i. The specimen should be stored refrigerated for transport,
   ii. Room temperature whole blood samples are acceptable. A disclaimer may be necessary (refer to specific enzymatic assay SOP for accepted samples).
   iii. Whole blood samples (ambient or refrigerated) need to arrive within 3 days from collection.

4. Suboptimal specimens: The medical director will determine whether a suboptimal specimen will be analyzed and whether the results will require a disclaimer. An ‘except test’ should be ordered to obtain approval from the client before reporting any results that require a disclaimer. Refer to CORP-PROC-7073, Specimen Handling: Ordering and Viewing Except Tests for instructions for ordering an ‘except’.

5. Unacceptable specimens:
   i. Blood sample that has been frozen.
   ii. Whole blood received after 3 days from collection
   iii. Sodium Heparin (green top) tubes, tubes containing no anticoagulant (plain red top) and serum separator tubes (SST) are NOT accepted.
   iv Grossly hemolyzed blood.

6. Sample Stability:
   Blood samples are acceptable for up to 3 days from the date of the draw.

Known Interferences

No known interferences have been described.

Reagents and Chemicals

NOTE: *If no expiration date is provided by the manufacturer, label the vial with an expiration date of 5 years from the date opened.*

1. BioRad protein assay kit II. BioRad Laboratories. Cat #500-0002
   a. BioRad Protein Assay Standard II–Bovine Serum Albumin (BSA). Store -20ºC.
   b. BioRad Protein Assay Dye Reagent Concentrate. Store 2-8 ºC.
2. Citric acid, monohydrate granular (C6H8O7:H2O), MW 210.14 VWR Scientific. Cat# JT0119-1. Store at ambient temperature.
3. Glycine ($CH_2(NH_2)COOH$), MW 75.07 VWR Scientific. Cat #97061-128. Store at ambient temperature.
4. Phosphoric acid. Sigma Cat# 452289-50mL. Store at room temperature in cabinet designed for only acids. Stable until expiration date or for two years from the date opened.
5. Methylumbelliferone Sodium Salt, MW 198.15 MP Biomedical. Cat #152475. Store at ambient temperature.
6. Methylumbelliferyl-beta-D-glucopyranoside MW 338.31. Sigma. Cat# M9766. Store at -20 ºC or below.
7. Sodium carbonate, anhydrous (Na_2CO_3) MW 105.9 JT Baker. Cat #3605-01. Store at room temperature.
8. Sodium phosphate, dibasic (Na2HPO4) MW 141.96 Sigma-Aldrich. ARUP# 44716. Store at ambient temperature
9. Sodium taurocholate MW 537.68 Sigma-Aldrich. Cat# T4009-250MG.
10. Tween-20 Sigma-Aldrich. Cat#P2287-100ML

Reagents Prepared In-House

1. Chilled Clinical Laboratory Reagent Water (CLRW).
    a. CLRW is produced by a CLRW purification system fed by a deionized water source.
    b. Store at 2-4ºC up to 24 hours.
2. Phosphate Citrate Buffer for beta-glucosidase assay (bGLU-PC buffer)
    a. Dissolve 5 g sodium phosphate (176 mmol/L) and 2.1g of citric acid (50 mmol/L) in 150mL of Nanopure water.
    b. Adjust to pH 5.4 with phosphoric acid.
    c. Add 2 mL of Tween-20 and 1.07 g of sodium taurocholate.
    d. Adjust the volume to 200mL with Nanopure water.
    e. Store in brown glass bottle refrigerated (2-4ºC) up to one year.
5. Glycine Carbonate Buffer (Alternatively, obtained Glycine Buffer from Reagent Lab Item #G-11060.)
    a. Dissolve 12.8g glycine (0.17mol/L) and 18.0g sodium carbonate (0.17mol/L) in 800mL of Nanopure water.
    b. Adjust the pH to 10.0 with 6M NaOH.
    c. Bring to final volume of 1000mL.
    d. Store in brown glass bottle at ambient temperature up to one year.
6. 4-Methylumbelliferone Standard Stock (1mmol) – Prepare in Amber bottle to protect from light
    a. Dissolve 3.96 mg of 4-methylumbelliferone in 19mL of Glycine Carbonate Buffer.
    b. Place on a stir plate 1 hour to thoroughly mix the reagent. Sonicate for 5 minutes prior to the 1 hour to help get the reagent into solution.
    c. After powder is dissolved, bring final volume up to 20mL.
    d. Store in brown glass bottle refrigerated (2-4ºC) up to 4 months.
7. 4-Methylumbelliferone Standard Working Solution (0.1mmol)
    a. Dilute 1 mL of 4-Methylumbelliferone Standard Stock (1mmol) in 9mL of Glycine Carbonate Buffer.
    b. Store in brown glass bottle refrigerated (2-4ºC) up to 4 months.
8. Methylumbelliferyl-beta-D-glucopyranoside (4-MUF) substrate
    a. The 4-MUF substrate must be freshly prepared each time.
    b. Dissolve the 4-MUF substrate in bGLU-PC buffer. Add 1.7mg (± 0.02) (5.0 mM) for each mL of substrate prepared. 1.6mL of substrate are required to run 6 samples (including blanks and controls): add 2.72 (± 0.06) of 4-MUF to 1.6 mL of bGLU-PC buffer.
    c. Sonicate for 10-30 seconds to dissolve if necessary.
    d. Place on ice until needed

Calibrators

1. Prepare standard curve using the **4-Methylumbelliferone Standard Working Solution** (0.1mmol) and add to the plate before reading samples.

   According to the table, below, pipette the appropriate amount (µL) of 4-methylumbelliferone Standard Working Solution (0.1mmol) into a tube designated for each level.

Calibrators	µL of 4-Methylumbelliferone Standard Working Solution	Final concentration µmol/L
1	0	0
2	3	0.12
3	13	0.52
4	26	1.03
5	54	2.0
6	65	2.53

2. Add 2.5mL of the GC buffer to each tube quickly. Use the bottle top dispenser set at 2.5 mL. Add the buffer directly into the tube. Be gentle but firm in adding the buffer, so that the calibrator will be properly mixed.

   Acceptance criteria: Standard 1 (calibration blank) reading < 50. Slope range should be evaluated when a new 4-methylumbelliferone Standard Working Solution is prepared. Slope range should be within mean slope ± 15%. Five replicates of the new standard curve should be read to establish the mean slope. Mean slope for the new standard should be compare to old slope, and be within ± 20%.

Control Samples

1. Control samples (normal and affected) are obtained from patient samples that were assayed for beta-glucosidase activity or healthy donors after IRB approval. See procedure below. Patient samples are de-identified according to ARUP protocols.
2. 75µL of the control sample (isolated leukocytes after sonication) is aliquoted into 0.5mL microcentrifuge tubes.
3. The microcentrifuge tubes are frozen at ≤-65°C for up to five years.
4. Control ranges are established evaluating the mean ± SD from at least 10 – 15 independent experiments.
5. If sample from an affected individual is not available, a normal sample can be used after heat-inactivation at 95 ºC for 3 minutes.

6. Alternatively, cultured fibroblasts from Gaucher disease patients can be used. The fibroblast pellet is re-suspended in water, and after sonication 75µL of the sample is aliquoted into 0.5mL microcentrifuge tubes. The microcentrifuge tubes are frozen at ≤-65°C for up to five years.

Equipment and Supplies

Laboratory Supplies

1. 13x75 Culture tubes, disposable, plastic (Sarstedt Cat#12860) ARUP Item #13652.
2. 96-well microplate for fluorescence, black, flat bottom. (VWR Cat#29442-070) ARUP Item#49998.
3. 96-well microplate, non-sterile, clear, flat bottom. (VWR Cat#29442-070) ARUP Item # 51701.
4. Adjustable volume pipettes and tips.
5. CoolRack® XT PCR96 (VWR Cat#89239-428)
6. Ice bucket with ice.
7. Microcentrifuge tubes, disposable (1.5mL) (VWR Cat#89000-028) ARUP Item #32863.
8. Microcentrifuge tubes, disposable (0.5mL) (VWR Cat#14231-060) ARUP Item #46212.
9. Microtubes. VWR Cat #211-0092.
10. PCR 8-Well Tube Strips. (VWR Cat #20170-004) ARUP Item #47813.
11. PCR 8-Well Tube Bubble Caps. (VWR Cat #20170-004) ARUP Item #47812.
12. Volumetric flasks (1000mL, 500ml, 100mL, 10mL).

Instruments and Consumables

1. BioRad DNAEngine® Peltier Thermal Cycler (PCR)
2. Eppendorf table top centrifuge 5417C.
3. Fisher Scientific Sonic Dismembrator Model 100.
4. Tecan Safire2™ 4-monochromator scanning fluorescence plate reader.

Records Required and Retention

Records are retained as described in the ARUP Corporate Record Retention Policy (CORP-POLICY-0160) which is in agreement with the Integrated Oncology and Genetics Division Record Retention Policy, IOG-01-1000 (generally 25 years for patient records). All work lists, run records, patient results, related documents, QC reports, and patient testing records generated during pre-analytic, analytic, and post-analytic processes

are kept on file in the laboratory for a minimum of two months (space permitting) and then may be transferred to an off-site storage. The raw electronic data for quality controls, and specimens should be backed up electronically on a monthly basis. Instrument records are stored in the laboratory for the life of the instrument and off-site an additional 7 years.

Instrument Calibration

The performance of the Safire2™ plate reader will be evaluated every month by the BCG lab by using the MultiCheck-Plus test plate. All data collected is retained by the BCG lab. Luminescence, absorbance, and fluorescence will be evaluated. The following optical tests are performed:

- **Fluorescence**
  - linearity
  - precision {SD}
  - signal/blank
- **Time-Resolved Fluorescence**
  - lag time check
- **Fluorescence Polarization**
  - precision{SD}
  - LED verification
- **Absorbance (NIST traceable)**
  - linearity
  - accuracy {bias}
  - precision {SD}
- **Luminescence**
  - repeatability
  - crosstalk
- **Optical alignment for all detection modes**
- **Excitation and Emission Wavelength (NIST traceable)**
  - accuracy {bias}
  - precision {SD}

Quality Control

- Two levels of controls for each sample type are available within a run: normal leukocytes and affected leukocytes. See Reagent Preparation section for making the controls.
- The beta-glucosidase activity must be within the defined limits. If this qualification is not met, the run will be reviewed by the Medical Director prior to acceptance and reporting of patient results.

Procedure
Isolation of Leukocytes

See specific SOP

Sample Preparation

1. Gather ice in an ice bucket.
2. Remove the tubes with the prepared white blood cell pellets from the freezer and place on ice. Use only one aliquot for patient. Retain the remaining aliquots in case there is a need to repeat the testing.
3. Loosen up the cell pellet by gently flickering the tube.
4. Remove the chilled nanopure water from the fridge and place on ice. Aliquot about 2mL of water in a tube to wash the sonicator probe tip before use, and between samples.
5. Add 500µL of chilled water (less if the pellet is very small, more if the pellet is large).
6. Immerse the sonicator probe tip into the tube until it is about ½ of the distance between the upper meniscus and the bottom of the tube.
7. Sonicate three times for 10 seconds at approximately 8-watt burst (Instrument setting of 3). Wait 10 seconds in between rounds of sonication taking the tip out of the sample. *Keep the sample on ice during the sonication.*
8. Between samples, wash the probe tip in water.
9. After sonication, quantify the protein concentration in the sample. The protein amount used for the assay should be between 0.010 mg and 0.035 mg (which corresponds to 35uL sample and a 0.3 – 1 mg/mL protein concentration). See section titled Protein Determination in Leukocytes.

Protein Determination in Leukocytes

1. Prepare a standard curve for protein determination using Protein Assay Standard II – Bovine Serum Albumin (BSA). BSA standard concentration is provided with each lot

of Protein assay Standard II received. Always record the BSA standard lot and its concentration.

2. Label 6 microcentrifuge tubes Cal 1-6. Pipet the appropriate amount of nanopure water and BSA standard or calibrator. Vortex briefly each tube.
3. BSA concentration in calibrator 6 is calculated based on the Protein assay Standard II lot received. In the chart below, an example using 1.48 mg/mL BSA

Calibrators	Concentration of BSA (mg/ml)	µL of BSA or Calibrator	µL of water
1	0	0	100
2	0.058	100 of Cal 3	200
3	0.175	100 of Cal 4	50
4	0.263	100 of Cal 5	50
5	0.394	100 of Cal 6	50
6	0.592	100	150

When BSA standard concentration = 1.48 mg/mL,
The Cal 6 BSA solution conc = (1.48 mg/mL x 100 uL) / (100 uL + 150uL)

= 0.592 mg/mL.

4. Label one microcentrifuge tube for each leukocyte sample. Transfer 20µL of each leukocyte sonicate into the labeled tube. Add 40 uL Nanopure water. Cap the tube and vortex briefly to mix.
5. Fill out a plate map, designating a calibrator, or specimen ID to wells of a clear 96-well plate.
6. Prepare the protein diluent in 15mL (or 50mL) conical tube
   a. Pipet 1 part Bio-Rad Protein Assay-Dye Reagent Concentrate plus 4 parts nanopure water as indicated in the chart below.

# of samples (Patients Plus Calibrators)	mL of Dye Reagent Concentrate	mL of Nanopure water
10	1	4
20	2	8
30	3	12

7. Pipette 10 uL of each calibrator and diluted patient leukocyte sonicates into the appropriate wells.
8. Using a repeat pipette with a 5 mL tip, add 200 uL protein diluent to each sample. Cover the plate and place the plate on orbital shaker. Shake the plate at 150 rpm for 5 minutes. The plate is then ready for protein measurement.
9. Turn on TECAN Safire2 and open the software Magellan6 (All programs>tecan>magellan6).
10. Choose “Start measurement” and continue.
11. Choose “Use predefined method”, click on method Protein **20140710.mth** and click on “make your selection”.
12. Name Workspace with assay and date information, such as “Protein 20140620.wsp”.
13. Place sample plate on cartridge with A1 on the upper left corner, and click on “Plate in” and start the run.
14. After the measurement, in the Edit dropdown list, click on “Copy to Exel”. An Excel sheet should pop up.
15. Save the data file again with the same file name. Discard the plate.
16. Exit Magellan and turn off TECAN Safire2.
17. Transfer data to the bGLU excel worksheet.

Assay

1. Controls
    a. Remove frozen aliquots of control samples from the freezer and place on ice to thaw.
    b. Remove frozen substrate from the freezer and place on ice to thaw.
2. Set up enough PCR 8-well tube strips to accommodate all samples into the cool rack.
3. Label each strip 1 to 12 according to the plate map. Each patient sample and control will require 2 wells each (i.e. 2 strips will accommodate the replicates for 8 samples).
    a. Two strips for each set of duplicate samples.
    b. Below an example of the plate map that includes six calibrators, blanks, positive and negative controls and 13 samples. Columns 1 to 12 represent the 8-well tube strips used. <u>Calibrators are NOT added into the strips; they are added to the plate few minutes before reading.</u>

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	S6 D1	S6 D2								Std. 1
B	Neg Cntr D1	Neg Cntr D2	S7 D1	S7 D2								Std. 2
C	Pos Cntr D1	Pos Cntr D2	S8 D1	S8 D2								Std. 3
D	S1 D1	S1 D2	S9 D1	S9 D2								Std. 4
E	S2 D1	S2 D2	S10 D1	S10 D2								Std. 5
F	S3 D1	S3 D2	S11 D1	S11 D2								Std. 6
G	S4 D1	S4 D2	S12 D1	S12 D2								
H	S5 D1	S5 D2	S13 D1	S13 D2								

4. Pipet 20µL of Nanopure water in use (chilled at 4°C) in the blank wells.
5. Pipet 20µL of the leukocyte sample or controls into each well. Briefly vortex the samples before adding them.
6. Add 80 uL of the substrate prepared fresh.
7. Cap all wells with bubble caps. Press well.
8. Briefly vortex the strips, and then place them on the PCR. Start the HOLD37_2 program: 120 minutes at 37.0°C, 30 seconds at 4.0°C; press yes for heated top.
9. Incubate for 120 minutes.
10. At the end of this 120 minutes incubation (about 10 minutes before the end), prepare the 4-Methylumbelliferone Standard calibration curve (See Calibration section).
11. At the end of the 120 minutes incubation, remove all samples from the PCR, take off the caps (carefully, to avoid sample splashing), and stop the reaction by adding 150uL of GC buffer. Use a multichannel pipette.
12. Transfer 40uL of the stopped reactions to a black microplate. Use a multichannel pipette, mixing samples before the transfer. Be gentle to avoid creating bubbles during the transfer.
13. Add 200uL of GC buffer to all wells of the microplate. Add 240uL of each calibrator to the microplate.
14. Turn on the plate reader (power button: ON). The green light will come on. Open the Magellan6 program. Be careful, the plate holder will come forward automatically. Set the plate in, placing the A1 well to the top left corner.
15. Read fluorescence on the Safire2 at 365 nm excitation and 441 nm emissions Select “start measurement”. Use the predefined method selecting the “Use Predefined Method” option. Select the following method: BCG HEX-A. Optimaze the Z-position in well A4 (using the standard 4). The program includes a brief shacking step to mix the samples before reading.
16. Transfer data to the bGLU excel worksheet (EDIT>Copy to excel; copy and paste data into the worksheet) and analyze the run.
17. Exit Magellan6 program and turn off the instrument.

Instrumentation Settings

- Instrument
  - Excitation (EX) 365nm
  - Emission (EM) 441nm
  - Excitation wavelength fixed mode (EX WL)
  - Slit
    - EX 10.0
    - EM 10.0
  - Measurement: Photometry
- Quantitation
  - Quantitation type = wavelength
  - Concentration nmol/mL
  - 1st order calibration
  - Force through zero
- Standards
  - 1 = 0
  - 2 = 0.12µmol/L
  - 3 = 0.52µmol/L
  - 4 = 1.03µmol/L
  - 5 = 2.0µmol/L
  - 6 = 2.53µmol/L

Acceptability of a Run

- The beta-glucosidase activity for each control (normal and affected) must be within the defined limits. If this qualification is not met, the run will be reviewed and, if appropriate, approved by the Medical Director prior to acceptance and reporting of patient results.
- The average fluorescence reading of the duplicate samples must be within 10% CV. If the CV is greater than 10%, the sample needs to be reviewed.

Result Interpretation & Reporting

The Medical Director is the sole person to interpret patient results and draw conclusions for specimen reporting. Other patient sample results within the reference interval may be verified in the LIS by the performing technologist without Medical Director's interpretation. The Medical Director may review patient results that do not fall within the reference interval.

Calculations

The beta-glucosidase activity is calculated for each sample:

Beta-glucosidase = nanomoles of substrate hydrolyzed per hour per milligram of protein

$$= \left[\left[\frac{(\text{fluor of total activity} - \text{fluor of blank}) * 0.24}{(y)} \right] * 6.25 \right] \div 2 \div (\text{mg of protein used for assay})$$

NOTE: The above calculations based upon:

/ 2 = correction of 120 minutes to 60 minutes

x 0.24 = correction to account for final concentration in samples versus calibrators

x 6.25 = correction for final volume

mg of protein used for assay = 0.020*protein conc. of sonicate (mg/mL)

Method Limitations

- Pseudo-deficiency has been shown for this enzyme, due to specific mutations affecting the exogenous substrate used by this assay, but not endogenous substrates. This can result in low enzymatic activity; although usually levels are higher than found in Gaucher patients.
- Enzymatic testing is not reliable for carrier detection.
- Further differentiation in subclinical types (Gaucher disease Type I, II or III) cannot be obtained by this test, and depends on clinical and/or molecular findings.

Back-Up Procedure

No back-up procedure is in place. If the test cannot be performed at ARUP Laboratories the sample will be sent out. Contact the Technical Supervisor and/or Group Manager who will, if appropriate, initiate the temporary referral process.

Referral Testing

If the test cannot be performed at ARUP Laboratories, the sample will be forwarded to Mayo Laboratories.

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Appendix

Appendix A: Reference Ranges
Appendix B: bGLU excel worksheet

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