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Mol Genet Metab. Author manuscript; available in PMC 2019 February 01.

Published in final edited form as:

Mol Genet Metab. 2018 February ; 123(2): 135–139. doi:10.1016/j.ymgme.2017.10.011.

Tandem mass spectrometry assay of β-Glucocerebrosidase activity in dried blood spots eliminates false positives detected in fluorescence assay

Pavlina Wolf[a], **Roy N. Alcalay**[b], **Christopher Liong**[b], **Emmaline Cullen**[a,c], **Michael W. Paciulo**[d], **William C. Nichols**[d], **Ziv Gan-Or**[e,f], **Wendy K. Chung**[g], **Tina Faulkner**[a], **Christopher Bentis**[a], **Robert J. Pomponio**[a], **Xiwen Ma**[a], **X. Kate Zhang**[a], **Joan M. Keutzer**[a], and **Petra Oliva**[a]

[a]Sanofi, P. O. Box 9322, Framingham, MA 01701

[b]Columbia University Medical Center, Neurological Institute, 710 West, 168th street, New York, NY 10032

[d]Division of Human Genetics, Cincinnati Children's Hospital Medical Center and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

[e]Department of Neurology & Neurosurgery, Montreal Neurological Institute and Hospital, McGill University, Montreal, QC, Canada

[f]Department of Human Genetics, McGill University, Montreal, QC, Canada

[g]Department of Pediatrics and Medicine, Columbia University Medical Center, New York, NY, USA

Abstract

Deficiency of β-Glucocerebrosidase (GBA) activity causes Gaucher Disease (GD). GD can be diagnosed by measuring GBA activity [1] Beutler and Kuhl 1990. In this study, we assayed dried blood spots from a cohort (n=528) enriched for *GBA* mutation carriers (n=78) and GD patients (n=18) using both the tandem mass spectrometry (MS/MS) and fluorescence assays and their respective synthetic substrates. The MS/MS assay differentiated normal controls, which included *GBA* mutation carriers, from GD patients with no overlap. The fluorescence assay did not always differentiate normal controls including *GBA* mutation carriers from GD patients and false positives were observed. The MS/MS assay improved specificity compared to the fluorescence assay.

Keywords

Dried blood spots (DBS); Gaucher Disease (GD); Newborn Screening (NBS); β-Glucocerebrosidase (GBA); Glucocerebroside; Lysosomal Storage Disorder (LSD)

Corresponding author: Petra Oliva, Phone: 1 508 270 2240, petra.oliva@sanofi.com.

[c]Present address: Great Ormond Street Hospital for Children, NHS Foundation Trust, Great Ormond Street, London, WC1N 3JH

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1. Introduction

Gaucher disease (GD; OMIM #230800, 230900, 231000) is an autosomal recessive lysosomal storage disorder (LSD) characterized by β-Glucocerebrosidase deficiency (GBA; EC 3.2.1.45; Acid β-D-Glucosidase; Glucosylceramidase) and the accumulation of its substrate Glucocerebroside (GL-1; Glucosylceramide) in lysosomes [2, 3]. The prevalence of GD ranges from ~1/1,000 live births in Ashkenazi Jewish populations to ~1:50,000 in the general population [4–6]. The progressive nature of GD and the availability of enzyme replacement and substrate reduction therapies create a need for early and accurate diagnosis of patients with GD [7–13]. However, the rarity and clinical heterogeneity of GD leads to diagnostic challenges [3, 14–17]. This study compared two assays used for screening GBA activity in DBS, the MS/MS assay and the fluorescence assay. The MS/MS assay was developed by Li et al and optimized by Zhang et al. [18, 19] and the fluorescence assay was originally developed by Chamoles et al. and optimized by Olivova et al. [20, 21].

Each assay employs a different synthetic substrate instead of the native GBA substrate Cn-Glucocerebroside (n=16–24) [22]. The MS/MS assay uses C12-Glucocerebroside, a synthetic analog of the native substrate with a shorter fatty acyl chain. The substrate is cleaved by GBA extracted from the DBS sample to produce a C12-ceramide product. The product is quantified against a C14-ceramide internal standard by MS/MS [18, 19]. The fluorescence assay utilizes the synthetic substrate 4-methylumbelliferyl β-D-glucopyranoside (4-MUG). GBA hydrolyses 4-MUG to release a fluorescent 4-methylumbelliferon (4-MU) product, which is quantified by fluorometer [20, 21]. The synthetic substrates are outlined in Figure 1.

2. Materials and Methods

2.1 Sample collection

Specimens were collected with written informed consent at Columbia University and at Sanofi Genzyme (Cambridge, MA) according to procedure established at Sanofi Genzyme [23]. The blood was drawn into 10 mL BD Vacutainer Blood Collection Tubes with K_2EDTA (Franklin Lakes, NJ) and 75 μL aliquots were spotted onto Whatman 903 filter paper (GE Healthcare, Piscataway, NJ). Blood spots were dried for a minimum of 4 h at room temperature. Dried sample cards were stored in sealed plastic bags at −20 °C with a desiccant and a humidity indicator. Cards were shipped at room temperature and stored at −80 °C until analysis.

2.2 Cohort Description and Clinical Status

The full cohort (n=528) contained control DBS (n=510) and GD DBS (n=18). Of these, 515 samples were collected at Columbia University in the “Spot” study, which tested the association between *GBA* mutations, GBA activity and Parkinson’s disease [24]. The 515 participants signed an informed consent to both enzyme activity analysis at Sanofi Genzyme and genotyping. The remaining 13 DBS samples came from Sanofi Genzyme collection of GD patients who consented to GBA enzyme activity analysis but not genotyping. After genotyping, the control group was further subdivided by *GBA* genotype into normal DBS

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(n=432) and carriers (n=78). The control group was defined as unaffected with respect to GD, but some individuals had Parkinson's disease as described in Alcalay et al. [24].

2.3 Genetic Testing

Samples from the 515 individuals with proper consent were genotyped in two ways. DNA was extracted using a standard salting-out method and all participants were genotyped for eight *GBA* mutations (p.N409S, p.L483P, p.L29Afs*18, ivs2+1G>A, p.D448G, p.V433L and p.R535H, and the p.[L483P; A495P; V499V] recombinant allele; legacy nomenclature: N370s, L444P, 84GG, ivs2+1G>A, D409G, V394L, R535H and the Rec*Nci*I recombinant allele, respectively) and two *GBA* variants (p.E365K and p.T408M, legacy nomenclature: E326K and T369M, respectively) as previously described [25, 26]. In addition, the *GBA* locus was fully sequenced in a second round of genotyping as described in Alcalay et al. [24]. The samples were blinded throughout GBA activity analysis.

2.4 GBA Enzyme Activity by Fluorescence Assay

GBA activity was measured as previously described [20] Briefly, one 3.2 mm-diameter punch from each DBS sample was extracted in 200 ;L 0.2 M citrate phosphate buffer, pH 5.2 containing 1% Triton® X-100 (Sigma, St. Louis, MO) and 1% sodium taurodeoxycholate (≥97% purity, Sigma, St. Louis MO) in a 96-well plate. Substrate solution (12.5 mM 4-MUG, Sigma, St. Louis, MO) was prepared either with or without inhibitor (0.5 mM Conduritol B Epoxide, Toronto Research Chemicals). Inhibited or uninhibited substrate solution was mixed 2:1 with DBS extract and incubated for 20 h at 37 °C. To stop the reactions, 100 μL of 0.5 M EDTA (pH 11.5) was added to each well. An eight point 4-MU standard curve (0 – 0.67 μM) was prepared on each plate in duplicate. The plate was read in a fluorometer at 355 nm excitation and 460 nm emission wavelengths. GBA activity was determined by subtracting the background activity measured in the inhibited reaction from that in the uninhibited reaction. Disease cut-off (1.71 μmol/L/h) was established as described in methods section 2.6. The limit of blank (LOB=0.16 μmol/L/h) and limit of detection (LOD=0.41 μmol/L/h) for the fluorescence assay were established previously [20].

2.5 GBA Enzyme Activity by MS/MS Assay

GBA activity was measured as part of an established MS/MS multiplex assay as previously described [18]. The GBA extract from one 3.2 mm punch per DBS sample was combined with C12-glucocerebroside substrate and C14-ceramide internal standard mixtures (The Center for Disease Control and Prevention, Atlanta, Georgia). Sealed 96-well plates were incubated on an orbital shaker at 37°C for 20 h and cleaned up according to the published protocol. Dried sample plates were stored at −20 °C. Prior to MS/MS analysis, plates were thawed and reconstituted with 200 μl of mobile phase (80:20 acetonitrile in water containing 0.2% formic acid). All analytes were monitored on an API 4000 triple quadrupole mass spectrometer (ABSciex, Framingham, Massachusetts, USA) by Multiple Reaction Monitoring (MRM). The enzyme activity of each sample was calculated from the ion abundance ratio of product to internal standard as measured by the mass spectrometer. Background activity of a blank filter paper was subtracted from the DBS activity. Activity was expressed as micromoles of product per liter of whole blood per hour (μmol/l/h). Two QC samples with previously established activity levels for each enzyme and disease positive

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samples were included in each plate as sample controls. The LOB=0.32 μmol/L/h and LOD=0.5 μmol/L/h for the MS/MS assay were established previously.

2.6 Statistical Analysis and Disease Cut-off

All statistical analysis was performed using the SAS v.9.4 software (SAS Enterprise, Cary, NC). A cut-off point distinguishing the control (normal + carrier) group and the GD group was derived by fitting a normal distribution to the control group ($F_{control}$) and the GD group ($F_{disease}$) using a direct estimation of each mean and standard deviation. The cut-off point $\chi_{cut\text{-}off}$ was then defined as the value with equal probability to occur in both, the control and the disease distribution:

$$f_{control}(\chi_{cut-off}) = f_{disease}(\chi_{cut-off})$$

where $f_{control}$ and $f_{disease}$ denoted the probability density function for $F_{control}$ and $F_{disease}$. Any value $\chi \leq \chi_{cut\text{-}off}$ was more likely to occur in the disease group distribution and any value $\chi > \chi_{cut\text{-}off}$ was more likely to occur in the control group.

The GD cut-off for the fluorosecence assay was established as 1.71 μmol/L/h. The GD cut-off for the MS/MS assay was established as 1.49 μmol/L/h.

3. Results and Discussion

The study cohort (n=528) contained control DBS samples (n=510) and GD samples (n=18). The full *GBA* gene was sequenced in consented samples (n=515) as described in methods. After genotyping, the control group was further subdivided into normal DBS (n=432) and *GBA* carriers (n=78). The carrier group included any heterozygous *GBA* sequence variation including non-coding mutations regardless of disease relevance. Table 1 outlines the cohort and genotyping results.

GBA activity was measured in all samples by both the MS/MS and the fluorescence assays. Activity results from the two assays are correlated in Figure 2. The mean activity of the normal and carrier DBS was significantly different ($p<0.0001$) Table 2, but neither assay could differentiate the carrier and normal DBS populations as expected. The correlation coefficient of the two assays R = 0.8073.

The mean GBA activity measured in normal controls, *GBA* carriers and GD DBS by the MS/MS and the fluorescence assays is listed in Table 2. Seven of the GD samples had activity levels below the LOD of the MS/MS assay (0.5 μmol/L/h), while all GD samples tested above the LOD of the 4-MU GBA assay (0.41 μmol/L/h). As expected, the GBA activity of all controls (normal DBS plus carriers) was significantly different from GD DBS activity in both assays ($p<0.0001$).

The GBA activity distribution span a greater range in the MS/MS assay compared to the fluorescence assay. The GBA activity measured by the MS/MS assay showed a clear separation between GD and normal control samples including *GBA* mutation carriers as shown in Figure 3. In contrast, the activity distribution measured by the fluorescence assay

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was continuous between the groups and false positives were observed. Specifically, eight *GBA* mutation carriers and two normal controls were tested below the GD cut-off of 1.71 μmol/L/h in the fluorescence assay. The GBA activity measured in carriers of specific mutations is reported in Supplementary Tables 1 and 2.

Both assays showed 100% sensitivity as they correctly detected all 18 GD samples as true positives. The MS/MS assay also showed 100% specificity, while the fluorescence assay specificity was slightly lower at 98.1%. The enzyme activity of *GBA* carriers clustered at the lower end of the normal activity distribution as observed previously [19, 21]. For *GBA* carriers in the MS/MS assay the 95% confidence interval (CI) = 2.7 – 13.1 compared to 5.7 – 18.5 for normal controls. For *GBA* carriers in the fluorescence assay the 95% CI = 1.0 – 4.4 compared to 1.8 – 6.0 for normal controls.

Assay characteristics such as reaction pH, time and temperature are similar in both assays. The difference in the specificity of the two assays may result from the structural differences of their respective synthetic substrates. The MS/MS assay uses a close structural analogue of the natural substrate. The ceramide moiety of the C12-Glucocerebroside substrate differs from the natural substrate only in the length of the fatty acid chain [19, 22]. In addition, the β-linked glucose head group known to bind the active site of GBA is flexible in the C12-Glucocerebroside and rigid in the 4-MUG substrate. This may affect the substrate enzyme configuration during GBA hydrolysis.

The difference between the fluorescence assay and MS/MS assay may be important to newborn screening and diagnostic laboratories [30–32]. False positives observed in GD screening with the fluorescence assay have been reported previously [33, 34]. False-positive test results can cause anxiety to patients and increase costs to the healthcare system. Therefore, borderline activity results in the fluorescence assay should be followed by genotyping.

4. Conclusion

This study demonstrated that the MS/MS GBA activity assay has higher specificity than the fluorescence GBA assay. Since other assay characteristics were similar in both assays, the structural features of the substrates might contribute to the observed differences. Both assays are suitable for GBA activity screening, but borderline results in the fluorescence assay should be followed by genotyping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this study provided by the Parkinson’s Foundation, the National Institutes of Health (K02NS080915 and UL1 TR000040, formerly the National Center for Research Resources, Grant Number UL1 RR024156), and the Brookdale Foundation. The authors would like to thank Karina Sakanaka BS and Paul Greene MD for patient referral and recruitment, and Ms. Judy Hull for facilitating the research collaboration.

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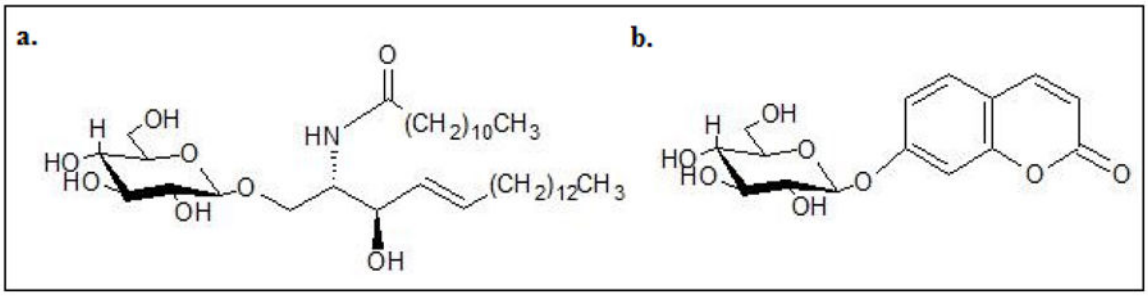

Figure 1. Synthetic substrates schema

a. C12-Glucocerebroside used in the MS/MS assay **b.** 4-methylumbelliferyl β-D-glucopyranoside used in the fluorescence assay.

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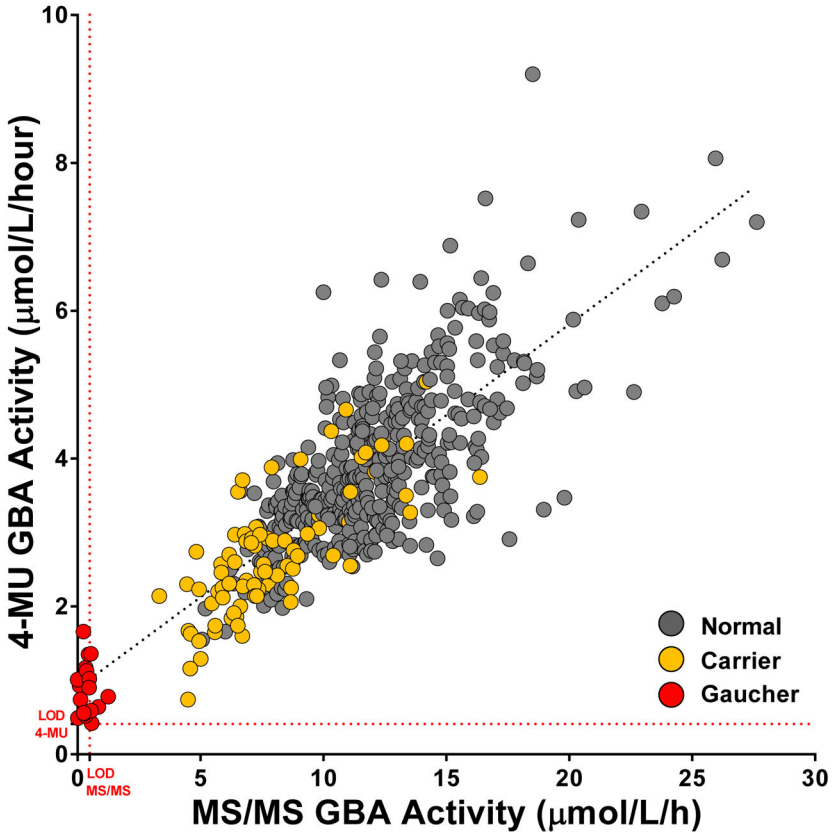

Figure 2. Fluorescence and mass spectrometry assay correlation

The GBA activity levels in control DBS (normal DBS plus *GBA* carriers) and GD DBS samples as measured by the MS/MS and the fluorescence (4-MU) assays. The red dotted lines indicates LOD for each assay as described in methods. R=0.8073.

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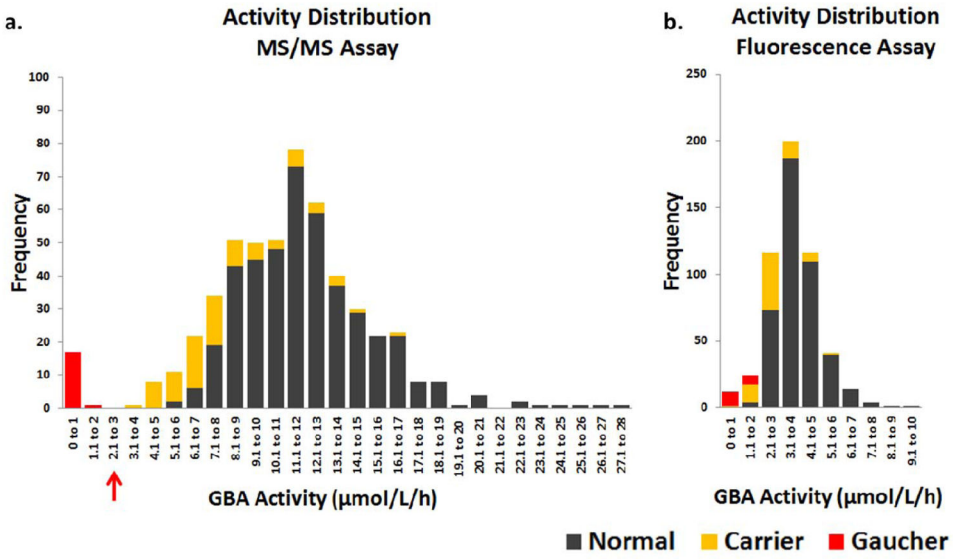

Figure 3. Frequency distribution of GBA enzyme activities in each assay

a. GBA activity distribution in the MS/MS assay. **b.** GBA activity distribution in the fluorescence assay.

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Table 1

Cohort description and genotyping results

DBS samples were divided by type (control and GD) and further subdivided by genotype (normal, carrier and Gaucher). As noted the genotyping was not performed for 13 of the GD patients from the Sanofi Genzyme collection. Variations in *GBA* sequence are described in legacy and HGVS nomenclature [27].

Sample		**Mutation or variant nomenclature**			**Count (n)**
Type	***GBA* Genotype**	**Legacy (name)**	**HGVS (protein)**	**HGVS (nucleotide)**	
Control n=510	**Normal n=432**	none detected in GBA sequence			432
	Carrier n=78	N370S	p.N409S	c.1226A>G	35
		L444P	p.L483P	c.1448T>C	4
		R496H	p.R535H	c.1604G>A	4
		n/a	n/a	c.(−203)A>G[1]	4
		84GG	p.L29Afs*18	c.84dupG	3
		ivs2+1G>A	r.(spl?)	c.115+1G>A	2
		K-27R	p.K13R	c.38A>G	2
		E326K	p.E365K	c.1093G>A	8
		T369M	p.T408M	c.1223C>T	7
		G202R	p.G241R	c.721G>A	1
		V294M	p.V333M	c.997G>A	1
		N392S	p.N431S	c.1292A>G	1
		T410M	p.T449M	c.1346C>T	1
		A456P	p.A495P	c.1483G>C	1
		F-37V	p.F3V	c.7T>G	1
		Q-8H	p.Q32H	c.96G>C	1
		L461P	p.L500P	c.1499T>C	1
		S110A	p.S149A	c.445T>G	1
Disease n=18	**Gaucher n=18**	N370S/N370S	p.N409S/p.N409S	c.1226A>G/c.1226A>G	4
		N370S/L444P+A456P	p.N409S/p.[L483P; A495P]	c.1226A>G/c.1448T>C; c.1483G>C[2]	1
		no genotype available			13

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[1] Suggested polymorphism in the promoter region of *GBA* gene that may result in lower promoter activity [28].

[2] Although the presence of c.1448T>C; c.1483G>C indicates a complex rearrangement with GBAP, additional substitutions were not observed in this patient [29].

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Table 2

GBA activity distribution, mean and standard deviation in μmol/L/h as measured by the MS/MS and fluorescence assays. $LOD_{MS/MS}$ = 0.5 (μmol/L/h).

Full Cohort n=528		GBA Activity (μmol/L/h)			
		MS/MS Assay		Fluorescence Assay	
		Range	Mean (SD)	Range	Mean (SD)
Control n=510	Normal n=432	5.07 – 27.63	12.11 (3.27)	1.55 – 9.20	3.90 (1.07)
	Carrier n=78	3.32 – 16.37	7.93 (2.65)	0.74 – 5.04	2.71 (0.85)
Disease n=18	Gaucher n=18	< LOD – 1.25	0.40 (0.30)	0.42 – 1.66	0.88 (0.35)