



Effect of sample collection on α -galactosidase A enzyme activity measurements in dried blood spots on filter paper

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ABSTRACT

Background: Fabry disease is an X-linked lysosomal storage disorder due to deficiency of *alpha galactosidase A* (AGAL, EC 3.2.1.22). Despite increasing utilization of dried blood spot (DBS) as samples for AGAL enzyme assays, the effects of blood sample collection techniques on enzyme activity have not been studied.

Methods: DBS samples were prepared by spotting blood collected into an ethylenediaminetetraacetic acid (EDTA) tube and by direct application of blood from a finger prick or a venipuncture syringe. AGAL activity was measured quantitatively by detecting the fluorescence of 4-methylumbelliferone (4-MU) generated using the substrate 4-methylumbelliferyl- α -D-glucopyranoside (4-MUGal) in an acidic pH for 20 h. N-acetyl-D-galactosamine (GalNAc) was used to inhibit α -galactosidase B (EC 3.2.1.49).

Results: We studied 88 previously diagnosed Fabry disease patients and 690 healthy controls. Average AGAL activity in DBS samples prepared using EDTA tubes was higher compared to those spotted directly irrespective of disease status.

Conclusions: The study confirms the need for collection method-specific reference ranges using DBS samples.

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1. Introduction

Fabry disease is an X-linked lysosomal disorder due to deficiency of *alpha galactosidase A* (AGAL, EC 3.2.1.22) enzyme activity resulting in multi-systemic organ dysfunction, including major manifestations of renal, neurological, and cardiac disease.

The true epidemiology of Fabry disease is unknown. The incidence of Fabry disease has been estimated at 1 in 44,000 to 55,000 male births. However, this figure may be a substantial underestimate of the true frequency [1]. A newborn screening study in Italy found an incidence of 1:3100 [2]. It is estimated that up to 12% of left ventricular hypertrophic cardiomyopathic patients may have undiagnosed Fabry disease [3]. Screening studies have demonstrated undiagnosed Fabry disease a variety of clinical settings: 15 of 508 (3%) hypertrophic cardiomyopathy patients [4], 21 of 432 males (4.9%) and 7 of 289 females (2.4%) with cryptogenic stroke [5], and 6 of 514 (1.2%) hemodialysis patients [6]. Fabry disease is diagnosed by measuring AGAL enzyme activity in leukocytes, plasma, or cultured fibroblasts [7]. However, some females with Fabry disease have normal levels of AGAL enzyme activity and may be missed if the AGAL enzyme assay is

used as a “stand alone” test for diagnosis or screening [8,9]. In contrast, nearly 100% of affected males are detected by measuring AGAL enzyme activity [10]. Molecular genetic testing is, therefore, the most reliable method to diagnose and screen for females with Fabry disease, but is not practical due to its high cost. Subsequently, many screening studies excluded females.

The use of dried blood spots on filter paper (DBS) for measuring AGAL activity in several diagnostic or screening centers [11–16] warrants the need for characterization and standardization of blood collection techniques. DBS can be made via direct blood spotting using a lancet or venipuncture syringe draw and from blood collected in a tube containing ethylenediaminetetraacetic acid (EDTA). The method of specimen collection may have an effect on AGAL enzyme assay. This is the first study to examine the effect of blood collection on AGAL enzyme activity in DBS.

2. Materials and methods

2.1. Reagents and equipment

4-Methylumbelliferyl- α -D-galactoside (4-MUGal), N-acetyl-D-galactosamine (GalNAc), 4-Methylumbelliferone (4-MU), EDTA, sodium taurocholate, and Corning 96-well black assay plates (non-binding) were from Sigma (St. Louis, MO). DMSO was from Mallinckrodt (Hazelwood, MO). Citric acid monohydrate, sodium hydroxide, and anhydrous dibasic sodium phosphate were from EMD Science (Gibbstown, NJ). Polypropylene 96-well microplate (non-binding), adhesive aluminum plate sealers,

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adhesive clear plate sealers, paper punch 1/8", Whatman #903 blood collection cards, hydrochloric acid, and HPLC grade water were from VWR (West Chester, PA). EDTA Vacutainer® tubes were obtained from BD (Franklin Lakes, NJ). A MaxQ Mini 4450 rocking platform was used for shaking (Barnstead International, Dubuque, IA); A Rotina 35R centrifuge was used for 96-well plate centrifugation (Hettich Zentrifugen, Tuttlingen, Germany); and a SpectraMax M2 fluorescent 96-well plate reader was used for measuring 4-MU (Molecular Devices Corp., Sunnyvale, CA).

2.2. Blood collection for Fabry disease and control cohorts

Specimens from 88 previously diagnosed Fabry disease patients (confirmed by mutational analysis) were collected with written informed consent. Specimens from 528 control adults were purchased from ProMedDx LLC (Norton, MA). Specimens from 162 control adults were collected at MGH (Massachusetts General Hospital, Boston, MA). Some blood was drawn into an ethylenediaminetetraacetic acid (EDTA) Vacutainer® tube designed for 1.8 mg EDTA per ml blood and stored at 4 °C. Within 24 h of collection (or as noted), the tubes were inverted several times to resuspend blood cells and 75 µl aliquots were spotted onto the filter paper. Other blood was spotted directly onto the filter paper post lancet finger stick or venipuncture syringe draw. Bloodspots were dried for at least 4 h at room temperature and were stored in sealed plastic bags at 4 °C for up to 1 week in a desiccated cabinet and at –20 °C with desiccant and a humidity indicator for longer periods.

For the collection technique study, 7 control male and 10 control female subjects were chosen randomly for DBS preparation by all collection methods (lancet direct, syringe direct and EDTA tube). Blood from an EDTA tube was spotted at 24, 48, and 72 h after storage at 4 °C. For the EDTA study, 2, 5, and 10 ml blood from 3 control females was drawn into three 100 cc Vacutainer® tubes containing EDTA for EDTA concentrations of 9, 3.6, and 1.8 mg/ml, respectively. DBS were prepared.

2.3. AGAL enzyme activity assay

AGAL activity was measured using a modification of the method of Chamoles [11]. For each sample, one 3.2 mm disk was punched from a DBS with sufficient clearance from the edge to ensure that the complete sample was saturated with blood. Each punch was placed into a separate well in 96-well plate and eluted in 250 µl of 1% sodium taurocholate (≥97% purity) at 4 °C for 1 h with shaking.

Substrate/inhibitor working solution (14 mmol/l 4-MUGal, 0.3 mol/l GalNAc) was prepared fresh daily by adding 100 µl of 0.7 mol/l 4-MUGal in DMSO to 4.9 ml of 0.31 mol/l GalNAc in water. Fifty microliters of substrate/inhibitor working solution, 100 µl of 0.07 mol/l citrate phosphate assay buffer at pH 4.5, and 50 µl of DBS eluate were combined in 96-well plates for enzyme reactions. In addition, blank reactions were prepared using 50 µl of substrate/inhibitor working solution and 100 µl of 0.07 mol/l citrate phosphate assay buffer at pH 4.5 without DBS eluate. Duplicate enzyme reactions and duplicate blank reactions for background subtraction were used for each sample.

Plates were sealed with adhesive aluminum plate sealers and incubated in a 37 °C water bath for 20 h. Reactions were terminated with 100 µl of 0.5 mol/l EDTA at pH 11.5. Fifty microliters of DBS eluate was added to each blank reaction well after termination. An 8-point 4-MU standard curve (0–1200 nmol/l) was prepared on each plate in duplicate by serial diluting a 4-MU stock solution (4 mmol/l in DMSO) in water. One hundred microliters of 0.5 mol/l EDTA at pH 11.5 was added to each standard curve well. The plates were read immediately in a fluorometer with 355 nm excitation and 460 nm emission wavelengths. Molar product quantities were calculated by linear regression from the standard curve. AGAL activity was determined using blank subtraction. DBS from a single venous blood specimen were included as internal QC samples on all plates. A detailed protocol is available upon request.

2.4. Statistics

Data analysis was performed using GraphPad Prism ver. 5.00 (San Diego, CA) and JMP version 7 software (SAS Institute, Cary, NC). Mean AGAL activity, SD, and 95% CI of mean were calculated for each group. The statistical analysis was performed using

Table 1
Precision evaluation results.

Sample representing	CV (%)
Sample 1 (female)	14.3
Sample 2 (female)	16.4
Sample 3 (female)	18.3
Sample 4 (male)	18.3
Sample 5 (male)	18.2
Sample 6 (female)	20.2
Sample 7 (male)	19.8
Sample 8 (male)	14.0
Average	17.4

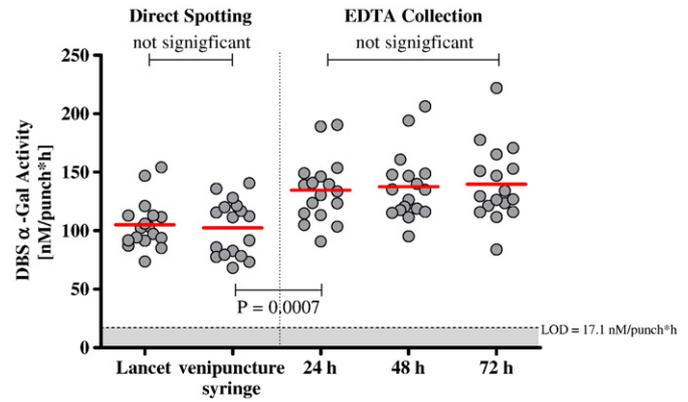


Fig. 1. Effect of blood collection methods on AGAL activity in control adults. The mean AGAL activity in DBS prepared from blood collected into EDTA tube was significantly higher than AGAL activity in DBS prepared by direct blood spotting ($p = 0.0007$) in all subjects. No difference was observed between direct blood spotting from venipuncture syringe draw or from lancet ($p = NS$). The mean AGAL activity remained relatively stable when the blood was spotted after storage at 4 °C for 24, 48 and 72 h.

parametric techniques. Means were compared by 2-tailed unpaired *t*-test or 1-way analysis of variance (ANOVA) with Tukey's post test and 95% confidence interval.

3. Results

3.1. Assay validation

Following Clinical and Laboratory Standards Institute (CLSI) guideline EP05 [17], assay precision was determined to be 17.4% by testing 8 samples from control adults (4 males and 4 females) in duplicate for 20 days by 2 operators. The CVs for samples are shown in Table 1.

The limit of detection (LOD) for AGAL activity was determined to be 17.1 nmol/l/punch/h by following the CLSI EP17 [18] guideline. The assessment was made with a proportion of false positive (α) to be less than 5% and false negative (β) less than 5% from 133 measurements (60 blank and 73 low-activity samples). Limit of blank (LOB) was calculated to be 8.1 nmol/l/punch/h.

3.2. Effect of blood collection method on AGAL activity in control adults

AGAL activity was measured in 17 DBS in the "collection technique study". The mean AGAL activity of samples prepared by direct blood spotting was significantly lower ($p = 0.0007$) compared to that of samples using blood collected into EDTA tube in all subjects (Fig. 1).

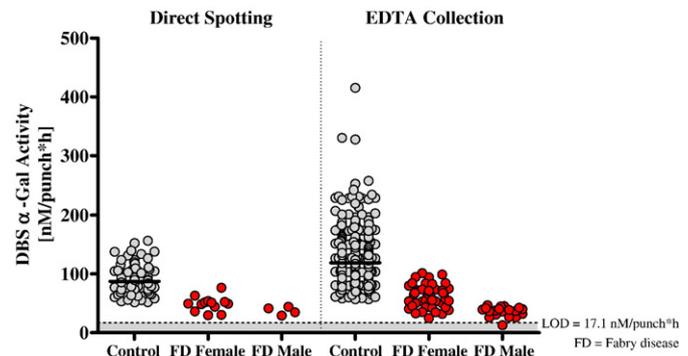


Fig. 2. Comparison of Fabry disease and control cohorts. Comparison of AGAL activity of 564 controls and 71 Fabry disease (FD) patients in DBS prepared from blood collected into EDTA tubes and AGAL activity of 126 controls and 17 Fabry disease (FD) patients in DBS prepared by direct blood spotting. Observed range AGAL activities for control and Fabry population are listed in Table 1. One male Fabry disease sample was below the LOD.



Fig. 3. Gender specific reference. Comparison of AGAL activities in DBS from 352 male controls and 212 female controls prepared from blood collected into EDTA tubes and AGAL activities in DBS from 55 male controls and 71 female controls prepared by direct blood spotting. Solid lines indicate medians. These groups were not significantly different and the lower confidence interval was unaffected by gender.

With the same subject, AGAL activity from blood collected in EDTA was up to 68% higher than blood spotted directly. By contrast, no significant difference was observed between using a syringe and lancet for direct blood spotting ($p = 0.73$).

Additionally, the mean AGAL activity remained relatively stable when the EDTA blood was spotted after storage at 4 °C for 24, 48 and 72 h; although, DBS prepared after 72 h had an increased CV of 23%.

AGAL activity was measured in 690 control adults (283 females and 407 males). The DBS for 564 control adults (212 females and 352 males) were prepared from blood collected into EDTA tubes. The rest of 126 DBS (71 females and 55 males) were prepared using direct blood spotting methods. The mean AGAL activity of DBS using EDTA collection is significantly different from that of DBS using direct blood spotting ($p < 0.0001$) (Fig. 2).

The multiple statistical analyses and a multiple statistical models were run in order to determine if differences exist between the genders in Fabry disease and control cohorts. The data was analyzed for outliers using box-plots, which is robust for normality. Many outliers existed in the male group. Log transformation significantly reduced outliers for the male groups, and added a few to the female groups. Overall, many more outliers were reduced by log transformation. Normality was checked (Shapiro–Wilk W test) on the data that was log transformed, outlier removed. Statistical t tests were performed on log transformed data. The groups were determined not to be statistically different. Gender did not influence the mean (Fig. 3).

The same transformed and outlier removed data sets were used for regression. There were no cases of a p value < 0.1 for slope, indicating no significant relationship between age and AGAL activity in DBS. R squared values were all < 0.1034 . In a mixed model analysis, age was not a significant factor, while sample collection type, and Non-disease/Fabry disease status were significant factors.

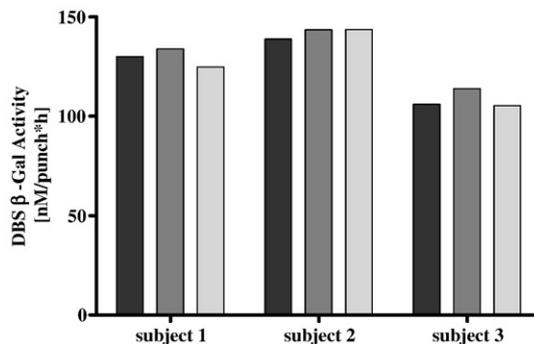


Fig. 4. Effect of EDTA concentration on AGAL activity. Comparison of AGAL activities measured when different volumes of blood were collected into the standardized EDTA tube in 3 control females. The results demonstrated that the concentration of EDTA did not affect either AGAL enzyme activity values or the accuracy of measurement.

Table 2

AGAL activity (nmol/l/punch/h) comparison between control cohort and Fabry disease patient samples collected in different methods.

	EDTA collection group			Direct spotting group		
	Control	Fabry disease Female	Fabry disease Male	Control	Fabry disease Female	Fabry disease Male
N	564	51	20	126	13	4
Mean	118.7	57.0	32.4	87.3	41.5	26.7
95% CI of mean	115.4–122	57.0–67.2	32.4–40.1	83.6–91.0	41.5–56.7	26.7–44.3
Minimum	57.2	24.9	13.1	52	30	29.2
Median	112	65.6	38.5	84.2	49.6	38.2
Maximum	415.4	101.3	46.7	156.6	76.6	44.3

3.3. Effect of EDTA concentration on AGAL activity

We studied the effect of the concentration of EDTA that results when different volumes of blood are collected into the standardized EDTA tube in 3 control females. DBS were prepared with 9, 3.6, and 1.8 mg/ml EDTA. The concentration of EDTA did not affect AGAL activity (Fig. 4).

3.4. Measuring AGAL activity in Fabry disease samples

DBS from 51 female and 20 male Fabry disease patients were made after collection into EDTA tubes; DBS from 13 female and 4 male Fabry disease patients were prepared using direct spotting methods. AGAL activity was measured in all samples and compared to sample collection method-specific control adults AGAL activity (Fig. 2 and Table 2). The AGAL assay was sensitive enough to differentiate samples from male Fabry disease patients and controls regardless the method of sample collection. AGAL activity from females with Fabry disease overlapped with the normal range as expected.

4. Discussion

Our study showed that the blood sample collection method used for DBS preparation influences AGAL activity. AGAL activity is higher using blood from EDTA tubes than direct blood spotting. However, the method for direct spotting collection, venipuncture vs. lancet, does not affect AGAL activity. This suggests that labs measuring AGAL activity in DBS should either use a single blood collection method or have collection method-specific reference ranges. The collection method should be included in the AGAL activity report. Of note, our results support no need for gender specific control references for Fabry disease screening since no difference of AGAL activity between genders in the control groups was found.

AGAL activities measured using blood collected with EDTA tubes were accurate, independent of the concentration of EDTA ranging between 1.8 and 9 mg/ml. Thus, small volumes of blood can be drawn

	EDTA concentration in final blood volume		
	9 mg/mL	3.6 mg/mL	1.8 mg/mL
subject 1	130.06	133.84	124.73
subject 2	138.87	143.55	143.61
subject 3	106.01	113.83	105.34

Values expressed in units of nM/punch*h.

■ 9.0 mg EDTA/mL of blood
 ■ 3.6 mg EDTA/mL of blood
 ■ 1.8 mg EDTA/mL of blood

and used to prepare DBS regardless of the size of the EDTA collection tube that is available.

The stability of AGAL activity in whole blood samples stored at 4 °C for up to 72 h after collection will permit clinicians who do not have access to the filter paper to send an EDTA tube of blood to testing labs. The lab can subsequently prepare the DBS for testing.

In conclusion, the results from all previously diagnosed male and many female Fabry disease patients were different than the respective control adult groups regardless of the method of sample collection. These results suggest that the fluorometric dried blood spot AGAL assay can be used as a diagnostic assay for Fabry disease. A large study to confirm the use of this test in diagnosing Fabry disease is currently underway.

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