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Blood spot versus plasma chitotriosidase: A systematic clinical comparison

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ABSTRACT

Objectives: This study aimed to evaluate the agreement between blood spot and plasma chitotriosidase using the economic substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside, and to investigate the utility of the blood spot assay for the wide scale screening for lysosomal storage disorders among the clinically suspected.

Design and methods: Blinded blood spot samples were compared with the corresponding plasma levels in 199 children (56 with confirmed diagnoses of ten different lysosomal storage disorders, 73 normal controls and 70 pathological controls). Several performance criteria (limit of detection, linearity, within-run and day-to-day precision and sample stability) were also evaluated.

Results: Plasma assay performed better by most criteria; however, blood spot performance was quite satisfactory. Quantitative values of the two methods can't be used interchangeably based on their 95% limits of agreement. Diagnostic sensitivity and specificity derived from ROC curves were 75.0 and 85.3% for the plasma assay and 71.4 and 79.0% for the blood spot assay, respectively. Cohen's kappa was 0.72 (95% CI: 0.616–0.821) denoting a good categorical agreement between the two methods.

Conclusion: The clinical use of blood spot chitotriosidase for the screening of lysosomal storage disorders can be quite practical, provided proper cut-off values are determined for each lab.

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Introduction

The chitotriosidase enzyme was the first discovered chitinase in humans. It was markedly elevated in plasma of patients with Gaucher's disease, a relatively common lysosomal storage disorder (LSD) [1]. Being produced exclusively by activated macrophages and polymorphonuclear leucocytes raised suspicion of its involvement in innate physiological immunity against chitin containing pathogens [2]. After initial discovery, its elevation was detected in many other LSDs [3–5], and many other non-lysosomal disorders; e.g. coronary heart disease [6], multiple sclerosis [7], β-thalassemia [8] and different types of infections [9,10]. Chitotriosidase activity in plasma is also established as a therapeutic monitor for enzyme replacement therapy in Gaucher's patients [11] and is under consideration to monitor therapy in other diseases such as Fabry [12], nephropathic cystinosis [13] and sarcoidosis [14].

Dried blood spot (DBS) on filter paper is a minimally invasive method for obtaining blood samples. In comparison with venipuncture, it is relatively easy and has a much lower cost for sample collection, transport and storage [15]. This is especially important in developing countries where specialized metabolic labs are rare and financial aspect is always an obstacle.

A method for assaying chitotriosidase activity in blood spots was first described using the substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside (4-MU-C3) [16]. Another substrate; 4-methylumbelliferyl-deoxychitobiose (4-MU-dC2), was later developed to avoid a flaw in the former one whereby, at optimal substrate concentration, glycolytic enzyme activity on 4-MU-C3 is hindered by its transglycosidase activity; the new substrate is not susceptible to this effect [17]. However, so far, 4-MU-dC2 is extremely expensive and not easily available; thus its utility in clinical practice is greatly minimized.

A limited comparison between blood spot and plasma chitotriosidase was previously conducted with 14 Gauchers patients and 12 healthy control subjects [18]. However, to our knowledge, no systematic comparison has been made. In this study, we tried to draw a full comparison between the two sample types by evaluating several performance criteria and a clinical comparison in 199 children, including some with LSDs as well as pathological and normal controls, seeking the level of agreement between the two methods with the clinically available substrate 4-MU-C3.

Abbreviations: DBS, dried blood spot; GM1, gangliosidosis M1; LSDs, lysosomal storage disorders; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; 4-MU, 4-methylumbelliferone; 4-MU-C3, 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside; 4-MU-dC2, 4-methylumbelliferyl-deoxychitobiose.

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Materials and methods

Subjects

Subjects included in this study were recruited from children attending different outpatient clinics at the Center of Social and Preventive Medicine (CSPM) and Abou Alreesh Children Hospital, Cairo University, Egypt. Over the period from December 2009 till August 2012, a total of 199 subjects were recruited: 56 children (39 males, 5.60 ± 3.55 years) with confirmed diagnoses of 10 different LSDs, diagnosed at the Inherited Metabolic Disorder Laboratory (IMDL) at the CSPM, and 143 matched control children; 73 (45 males, 5.30 ± 3.60 years) with minor non-related complaints and normal routine laboratory results were considered as normal controls and 70 (44 males, 5.60 ± 4.40 years) with preliminary symptoms that were confusing with some LSDs, but diagnosed otherwise. The latter group was considered as pathological controls and was further divided according to major complaint into three subgroups: 31 with neurological manifestations, 22 with hepatosplenomegaly and 17 with idiopathic cardiomyopathy after excluding cardiomyopathy-causing LSDs (Table 1). Another 23 LSD patients, diagnosed at our laboratory during the study period, were excluded due to unavailability of either plasma or blood spot samples. The study was approved by the institutional review board at Cairo University Children's Hospital, and written informed consents were obtained from parents of patients.

Samples

One to two milliliter of blood was collected from each subject, mixed appropriately with EDTA, and then 50 μL aliquots were spotted on Whatman 903 filter paper (Whatman Inc. USA), while the remainder was centrifuged for plasma collection. Blood spot cards were left at room temperature until fully dried, then put in tightly sealed aluminum bags and kept at −80 °C together with plasma samples till time of analysis. Blood spots were later coded and blinded from the operator who also performed the plasma assay. Tests were assayed in duplicate for both sample types.

Chitotriosidase assays

Chitotriosidase in plasma was measured based on the method of Hollak et al. [1]. Ten microliter of plasma was mixed with 100 μL of 0.022 mmol/L 4-MU-C3 (Sigma) in Citrate/Phosphate buffer, 0.1/0.2 mol/L, pH 5.2 and incubated at 37 °C for 15 min. The reaction was stopped with 2 mL of 0.5 mol/L Carbonate/Bicarbonate buffer, pH 10.7.

Chitotriosidase in blood spots was measured according to Chamoles et al. [16]. A 3.2 mm dried blood spot punch was mixed with 20 μL of 0.19 mmol/L of 4-MU-C3 in distilled water and 20 μL of 0.5 mol/L acetate buffer, pH 5.0. Incubation was performed at 37 °C for 30 min and the reaction was stopped with 300 μL of 0.5 mol/L Carbonate/Bicarbonate buffer, pH 10.7.

Fluorescence activity was measured by FP 6200, Jasco, Tokyo, Japan; Excitation: 365 nm, Emission: 448 nm. Enzyme activities were calculated based on a calibration curve of 4-methylumbelliferone (4-MU) for each assay. Plasma samples exceeding determined linearity were diluted in Citrate/Phosphate buffer; while blood spot dilution was performed by manipulating the acetate buffer volume.

Performance criteria

Limit of detection

Determination of the Limit of detection was conducted through assaying 5 blank samples and 5 low-level samples in duplicate over 5 analytical days following the non-parametric approach [19]. Blank samples were taken from suspected enzyme deficient individuals, and low-level samples were below 10 nmol/mL/h for both sample types.

Linearity

Linearity curves were constructed by serial dilution of both sample types obtained from a Gaucher's patient and plotting the measured against the expected concentration of each dilution. All levels were tested in duplicate and the average was taken. Linearity experiments were repeated twice on two separate days.

Precision

Within-run and day-to-day precision studies were performed for both assays through the analysis of 3 concentration levels (Level 1: normal, Level 2: mildly elevated and Level 3: markedly elevated). Each level was assayed in duplicate for 20 analytical days over a 2 month period. Within-run and day-to-day precision values were evaluated based on the duplicates and first replicates of each day, respectively [20].

Sample stability

Evaluation of sample stability was done by aliquoting plasma and blood spots of a normal control. Aliquots were preserved at different temperatures: 37 °C, room temperature (RT: 20–25 °C), 4 °C and −80 °C, and measured repeatedly in duplicate over a 4 month period. Any plasma or blood spot sample crossing the limits determined by its base line level ±2 standard deviations of the corresponding sample in

Table 1
Study subjects and levels of chitotriosidase enzyme assays.

	n	Plasma chitotriosidase		Blood spot chitotriosidase	
		(High/total)[a]	Median (range), nmol/mL plasma/h	(High/total)[a]	Median (range), nmol/mL blood/h
Normal controls	73	3/73	13.2 (0–72.3)	6/73	9.7 (0–51.7)
Pathological controls	70	18/70	15.6 (0–1059)	24/70	16.1 (0–974)
Neurological	31	6/31	13.6 (0–92.4)	7/31	14.9 (0–57.2)
Hepatosplenomegaly	22	10/22	32.0 (0.9–1059)	13/22	39.5 (0–974)
Cardiomyopathy	17	2/17	7.0 (0–45.3)	4/17	9.0 (0–94.3)
Patients	56	42/56	116 (0–8156)	40/56	64.3 (0–4410)
Gaucher	21	21/21	2786 (1235–8156)	21/21	1584 (476–4410)
Maroteaux-Lamy (MPS VI)	8	4/8	30.5 (0–118)	4/8	15.6 (0–62.7)
Metachromatic leucodystrophy (MLD)	5	4/5	147 (18.1–277)	4/5	35.1 (21.5–139)
Hurler–Scheie (MPS I)	5	2/5	9.7 (8.8–38.4)	1/5	8.0 (4.5–31.1)
Morquio (MPS IV)	5	4/5	63.5 (7.8–115)	4/5	27.2 (12.7–102)
Pompe	4	2/4	31.8 (0–129)	1/4	11.5 (0–25.7)
San Filippo (MPS III)	4	1/4	8.8 (0.2–67.2)	1/4	1.9 (0–23.6)
Fabry	2	2/2	56.5, 53.1	2/2	39.3, 29.3
Niemann–Pick A/B	1	1/1	837	1/1	675
Gangliosidosis M1 (GM1)	1	1/1	172	1/1	90.5

[a] Chitotriosidase (high/total) was based on cut-off values determined by optimal sensitivity and specificity derived from ROC curves.

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