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Development and application to clinical practice of a validated HPLC method for the analysis of β-glucocerebrosidase in Gaucher disease

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ABSTRACT

The main objective of our study is to develop a simple, fast and reliable method for measuring β-glucocerebrosidase activity in Gaucher patients leukocytes in clinical practice. This measurement may be a useful marker to drive dose selection and early clinical decision making of enzyme replacement therapy. We measure the enzyme activity by high-performance liquid chromatography with ultraviolet detection and 4-nitrophenyl-β-D-glucopyranoside as substrate.

A cohort of eight Gaucher patients treated with enzyme replacement therapy and ten healthy controls were tested; median enzyme activity values was 20.57 mU/ml (interquartile range 19.92–21.53 mU/ml) in patients and mean was 24.73 mU/ml (24.12–25.34 mU/ml) in the reference group, which allowed the establishment of the normal range of β-glucocerebrosidase activity.

The proposed method for leukocytes glucocerebrosidase activity measuring is fast, easy to use, inexpensive and reliable. Furthermore, significant differences between both populations were observed ($p = 0.008$). This suggests that discerning between patients and healthy individuals and providing an approach to enzyme dosage optimization is feasible. This method could be considered as a decision support tool for clinical monitoring.

Our study is a first approach to in depth analysis of enzyme replacement therapy and optimization of dosing therapies.

1. Introduction

Gaucher disease (GD) is an autosomal recessive, lysosomal storage disease caused by deficiency of acid β-glucocerebrosidase (GBA) which results in secondary accumulation of glucocerebroside within the macrophage lysosomes. This storage disease produces a multisystem disorder involving the liver, spleen, bone marrow, skeleton, lungs and occasionally the central nervous system. Three major clinical subtypes have been described: type I GD is the chronic nonneuropathic form which is the common clinical variant; type II is the infantile neuropathic form; and type III is the juvenile neuropathic form [1].

Recently, two non-lysosomal neutral GBA have been identified: GBA2 [2,3], known as a bile acid β-glucocerebrosidase and GBA3 [4] known as a klotho-related protein (KLrP). Physiological roles of the two enzymes remain to be elucidated.

Nowadays, GBA activity measurement is used for diagnosis of GD, showing tissue glucocerebrosidase deficiency, especially

Abbreviations: GD, Gaucher disease; GBA, β-glucocerebrosidase; GlcCer, glucocerebroside; FACS, fluorescence-activated cell sorter; PNP-Glc, 4-nitrophenyl-β-D-glucopyranoside; PNP, p-nitrophenol; ERT, enzyme replacement therapy; IMG, imiglucerase; EMA, European Medicines Agency; HPLC–UV, high performance liquid chromatography with ultraviolet detection; Tris, Tris-(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetraacetic acid; ACN, acetonitrile; U, unit; MLR, Multiple linear regression; FDA, Food drugs and administration.

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in leukocytes and fibroblasts [5]. Using a physiologic glycolipid substrate, glucocerebroside, radiolabeled in either glucose or the long-chain fatty acid moiety, makes the method to determine β-glucocerebrosidase (GBA) activity time-consuming, expensive and not readily applicable to usual clinical laboratory setting.

Beutler and Kuhl [6] were the first ones to demonstrate the usefulness of the fluorogenic, nonphysiologic substrate 4-methylumbelliferyl-β-D-glucopyranoside for the diagnosis of GD and carrier identification, using leukocytes as a source of glucocerebroside (GlcCer). Lorincz et al. [7] developed a quantitative fluorescence-activated cell sorter (FACS) assay for the determination of relative GBA activity in monocytes by using CD14. The assay is easy to use, sufficiently sensitive to distinguish GBA activity found in gaucher patient monocytes from that in normal controls and shows excellent correlation with the standard method [6], but a FACS's disposal is needed.

In an other study [8] an HPLC-based assay using fluorescent GlcCer analogs [glucosylceramide, (4-nitrobenzo-2-oxa-1,3-diazole)-GlcCer (C6-NBD-GlcCer) and (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-labeled GlcCer (C12-BODIPY-GlcCer)] has been developed to detect GBA activity in human fibroblasts. This method for GBA activity determination is fast, reliable and specific and it clearly showed a decrease of GBA activity in GD patient fibroblasts and serum compared with healthy individuals, suggesting that the method is applicable for the GD diagnosis.

Recently, 4-nitrophenyl-β-D-glucopyranoside (PNP-Glc) has been proposed as synthetic substrate for GBA activity monitoring in GD type I human serum with a spectrophotometric analytical assay performed at Shire Human Genetic Therapies, Inc (Cambridge, MA) [9]; this assay releases p-nitrophenol (PNP), which has a characteristic absorbance at 315 nm.

Additionally, macrophage-targeted enzyme replacement therapy (ERT) [10] ameliorates anemia and thrombocytopenia, decreases organomegaly and may improve or prevent the progression of bone disease [11–13]. Two different recombinant β-glucocerebrosidase preparations are in use for treatment: Imiglucerase (IMG) (Cerezyme®, Genzyme Corporation, Cambridge, MA) is a recombinant infusible enzyme produced in chinese hamster ovary cells, approved by Food and Drug Administration (FDA) at 1994 [14]. Velaglucerase alfa is an investigational human enzyme produced in a human cell line using proprietary Gene Activation technology (Shire Human Genetic Therapies Inc, approval in February 2010) [15]. The initial recommended dose is 60 U/kg body weight every two weeks. Nowadays, professional association guidelines, based on clinical experience, recommend to chose the initial dose based exclusively on the patient clinical situation [16]: bone or lung disease, 60 U/kg/every other week; visceromegalies or anemia and/or thrombocytopenia only, 30–60 U/Kg/every other week. It is not recommended to decrease dosing regimens below 15 U/kg/every other week.

Therefore, dose selection and clinical decision making are based on clinical parameters and markers monitoring without clear evidence of the disease control grade. Accordingly, searching for new markers to help us in dose adjustment is an ongoing challenge for clinicians.

In an article about pharmacokinetic analysis of ERT based on serum levels [9], it was demonstrated that the maximum serum concentration and the area under the curve were linearly proportional to dose, there was a proportionality between dose and enzyme activity in serum and there was individual variation between patients. The decision regarding the appropriateness of therapeutic drug monitoring is based on these considerations; therefore GBA activity in patients treated with ERT seems to be a good candidate for clinical pharmacokinetic monitoring. In that case GBA activity could be employed for ERT dose adjustment.

The objective of the current study is to develop a simple, fast and reliable method for estimation of GBA activity based on high-performance liquid chromatography (HPLC) with ultraviolet detection and PNP-Glc as substrate. We present the results of an HPLC–UV assessment of residual GBA activity in a cohort of patients with GD type I treated with ERT and healthy controls (reference group), to demonstrate that this method is a useful tool for therapeutic drug monitoring in clinical practice.

2. Materials and methods

2.1. Study

It is a prospective and observational study with paired groups carried out during two years (from January 2009 to December 2010).

The study was divided into two phases:

- *Phase I (research study)*: development and validation of the analytical method for measuring GBA activity in leukocytes by HPLC–UV.
- *Phase II (clinical study)*: establishment of the normal range of GBA activity in leukocytes of the reference group and monitoring the GBA of patients with GD type I before starting the infusion of IMG.

2.2. Patients and reference group

Healthy volunteers (reference group) without a known genetic or chronic disease were selected for the study in order to know the normal scope of GBA activity. They did not receive any medication during the time of the study.

Patients (test group) at least 18 years of age with confirmed diagnosis of Gaucher disease based on clinical and biochemical criteria (documented deficient of GBA by enzyme assay) were eligible for this study if they met imiglucerase therapy with stable dose and interval at least 6 months prior to enrollment.

Research protocols and consent forms as well as the overall investigation were ethically and scientifically approved by the Medical research and Ethical Committee of Doctor Peset University Hospital (Code CEIC 50/10). All the members of reference and tested groups were previously informed about the study and consented to participate in it.

2.3. ERT with IMG

IMG (Cerezyme®) was supplied as a lyophilized product and shipped at 2–8 °C. The product was reconstituted with preservative-free sterile water for injection and slowly mixed with NaCl 0.9% to a final volume of 250 ml. The final solution of IMG was intravenously administered across a 0.2 μm filter for 60 min.

Patients included in the study received a perfusion of IMG (Cerezyme®) with a stable dose of 20–60 U/kg every other week or every four weeks, for at least 6 month.

2.4. Reagents

4-Nitrophenyl-β-D-glucopyranoside (PNP-Glc), 4-nitrophenol (PNP), conduritol-β-epoxide (CBE) and 0.5 M ethylenediaminetraacetic acid (EDTA) disodium salt solution were purchased from Sigma–Aldrich (St. Louis, USA). PBS buffer was purchased from Beckman Coulter (France). Tris-(hydroxymethyl)-aminomethane (Tris) and sodium chloride (NaCl) were purchased from Scharlab (Barcelona, Spain). NaCl 0.9% was purchased from Baxter (Barcelona, Spain). Potassium dihidrogen phosphate was obtained from Merck (Germany); acetonitrile (ACN) and methanol were