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A new method for the measurement of lysosomal acid lipase in dried blood spots using the inhibitor Lalistat 2

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ABSTRACT

Background: Cholesterol ester storage disease (CESD) and Wolman Disease (WD) are due to deficiency of lysosomal acid lipase (LAL). A new method is described for the measurement of LAL in dried blood spots (DBS) using Lalistat 2 an inhibitor of LAL.
Methods: LAL activity in DBS extracts was measured using the substrate 4-methylumbelliferyl palmitate. LAL activity was determined by measuring total lipase activity and lipase activity in the presence of Lalistat 2. The specificity of Lalistat 2 was investigated using human recombinant LAL (hrLAL) and human pancreatic lipase (hPL).
Results: Lalistat 2 inhibited hrLAL with 1% residual activity at 1 μM inhibitor but had no effect on hPL up to 10 μM. LAL activity in DBS samples obtained from normal controls ($n=140$) was 0.50–2.30 nmol/punch/h and in patients with CESD was <0.03 nmol/punch/h ($n=11$). Activity in carriers showed intermediate activity: 0.15–0.40 nmol/punch/h ($n=15$).
Conclusions: Measurement of LAL using DBS is made difficult by the presence of other lipases in whole blood. Lalistat 2 is a specific inhibitor of LAL which allows the determination of LAL in DBS. Results show the method differentiates clearly between normal controls, carriers and affected cases.

1. Introduction

Wolman disease (WD) and Cholesterol ester storage disease (CESD) are lysosomal storage disorders due to deficiency of the enzyme lysosomal acid lipase (LAL; EC 3.1.1.13). The enzyme cleaves both cholesterol esters and triglycerides under acidic conditions. WD is the severe form of the disorder presenting in infancy with hepatosplenomegaly, adrenal calcification and failure to thrive. CESD is most commonly identified in childhood or adolescence with hepatomegaly, premature cardiovascular disease and non alcoholic steatohepatitis (NASH) [1]. The incidence of CESD is estimated from genetic studies to be 1:40,000 [2], though clinically far fewer cases than this are identified. The incidence of WD is estimated at approximately 1:500,000 [3]. There is no specific treatment for WD with death normally occurring by 3 to 6 months. Current therapy for CESD consists of a low-fat diet and lipid-lowering drugs such as statins and colestyramine [4]. There is interest in the potential use of recombinant human LAL for enzyme replacement therapy (ERT) in LAL deficient patients [5] and the efficacy of recombinant human LAL has been demonstrated in an LAL-null mouse model [6].

A method has been adapted for the measurement of LAL in DBS based on the method of Guy et al. [7] applied originally to the measurement of LAL in fibroblasts. LAL is measured using the fluorimetric substrate 4-methylumbelliferyl palmitate (4 mU palmitate) with cardiolipin present as an activator of LAL. The presence of other forms of lipase in whole blood [8] will interfere with the measurement of LAL. Lalistat 2 is a specific inhibitor of LAL [9,10] and by measuring total lipase activity and lipase activity in the presence of Lalistat 2 LAL can be determined in DBS.

Lalistat 1 is an analogue of Lalistat 2. They are thiadiazole carbamates with similar inhibitory effect on LAL [10]. Both compounds were assessed in our assay and Lalistat 2 found to give marginally higher activity for LAL at the same concentration of inhibitor. Therefore Lalistat 2 was selected as the inhibitor of choice in the assay.

2. Materials and methods

2.1. Samples

A reference range was established using anonymised ethylenediaminetetraacetic acid (EDTA) blood obtained from samples submitted to our laboratory for routine diagnostic testing. Blood was spotted on to filter paper (Whatman grade 903 Schleicher & Schuell) on the day of venepuncture and allowed to dry overnight at room temperature. Samples were stored double-bagged with dessicant at −20 °C and analysed within 1 week of storage. DBS samples from 11 patients with CESD and 15 carriers were provided by Synageva BioPharma

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(Lexington, Massachusetts, USA). Samples were obtained following written, informed consent.

2.2. Sample collection and storage for stability study

EDTA blood obtained from 3 healthy adult controls was used to prepare DBS samples. Samples were stored at room temperature, 4 °C and −20 °C doubled-bagged with dessicant for up to 100 days.

2.3. Chemicals and reagents

Cardiolipin (sodium salt, bovine), sodium acetate (trihydrate), 4-methylumbelliferone (4 mU), dimethylsulfoxide (DMSO), human serum albumin (HSA) and hPL were obtained from Sigma–Aldrich Company Ltd. (Dorset, England). 4 mU palmitate was from Apollo Scientific Ltd (Stockport, Cheshire, UK). Mercuric chloride was from Merck KGaA (Darmstadt, Germany). Triton X-100 was from Koch Light (Cambridge, Cambridgeshire, UK). Lalistat 1 and Lalistat 2 were donated by Dr. P Helquist, Chemical Tools (South Bend, IN, USA) [10]. Recombinant human LAL was supplied by Synageva Biopharma (Lexington, Massachusetts, USA).

A buffer solution was prepared using 0.15 M acetate buffer pH 4.0, 1.0% Triton X-100. 14 ml buffer was retained at 37 °C (water bath) and to this was added 1.0 ml 0.5% (w/v) cardiolipin in methanol and 400 µl 13.3 mM 4 mU palmitate in DMSO (heated to 55 °C to dissolve) to give substrate-buffer solution. 30 µM Lalistat 2 was prepared fresh each time by diluting 200 µM Lalistat 2 (in DMSO) with distilled water. A 30 µM working solution of Lalistat 1 was prepared from 200 µM stock in the same way.

2.4. Sample extraction and assay procedure

3.2 mm spots were extracted with 200 µl water by mixing for 1 h at room temperature. The assay was performed using 96 well plates (Optiplate 96, Perkin Elmer, Cambridge, Cambridgeshire, UK). Uninhibited and inhibited reactions were each performed in duplicate requiring 4 wells per sample. 40 µl sample was added to each well and 10 µl 30 µM Lalistat 2 was added for inhibited reaction. 10 µl water was added to corresponding wells for uninhibited reaction. The plate was sealed with adhesive aluminium film and placed on a plate shaker for 2 min followed by pre-incubation for 10 min at 37 °C. 150 µl substrate-buffer solution (retained at 37 °C until use) was added to all wells and the plate re-sealed. The plate was shaken for a further 2 min and incubated at 37 °C for 3 h. Reaction was stopped by adding 100 µl 15 mM $HgCl_2$ to all sample wells.

A 0–2.5 nmol 4 mU standard curve was made by diluting 0.5 mM 4 mU standard with water (200 µl per well) and adding 100 µl 15 mM mercuric chloride to each well. Fluorescence was measured using a Wallac Victor 2 1420 Multilabel Counter (Perkin Elmer, Cambridge, Cambridgeshire, UK) (excitation 355 nm, emission 460 nm). The instrument was programmed to read the plates twice with a 5 minute interval between readings to allow dark adaptation of 4 mU. The second reading was used to determine results. LAL activity was determined by subtracting activity in the inhibited reaction from uninhibited reaction (total lipase) and calculated as nmol/punch/h.

3. Results

The effect of Lalistat 2 as an inhibitor of LAL was assessed by incubating Lalistat 2 with rhLAL (0.002 U/ml). Lalistat 2 was shown to inhibit rhLAL with only 1% residual enzyme activity remaining at a concentration of 1 µM inhibitor (Fig. 1). The effect of Lalistat 2 on hPL (6 U/ml) was assessed and found to have no effect on enzyme activity up to 10 µM inhibitor (Fig. 2).

The effect of Lalistat 1 and Lalistat 2 were compared by assessing enzyme activity using a pooled DBS extract from the same sample.

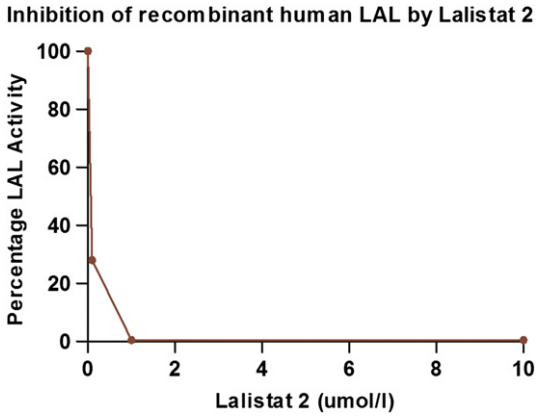

Fig. 1. Inhibitory effect of Lalistat 2 on rhLAL. Final concentration of rhLAL 0.002 U/L. Enzyme pre-incubated with inhibitor for 10 min. Incubation time 10 min.

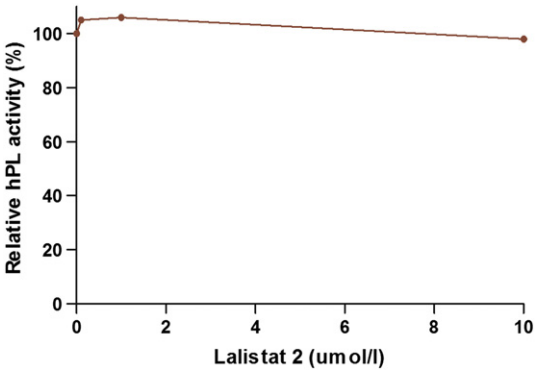

Fig. 2. Inhibitory effect of Lalistat 2 on human pancreatic lipase. Final concentration 6 U/ml. Enzyme pre-incubated with inhibitor for 10 min. Incubation time 2 h.

Inhibitors were present at concentrations from 0.5 to 5.0 µM (Fig. 3). Activity derived for LAL in DBS extract was higher with Lalistat 2 than Lalistat 1 at all concentrations of inhibitor (mean difference 7%) demonstrating more effective inhibition of LAL with Lalistat 2 than Lalistat 1. Optimal inhibition is achieved at a concentration 1.5 µM for both inhibitors.

The effect of pre-incubating enzyme with inhibitor was assessed by incubating rhLAL with Lalistat 2 for up to 15 min before adding substrate. Lalistat 2 was present at 0.1 to 10 µM. The degree of inhibition was dependant on both the concentration of inhibitor and pre-incubation time (Fig. 4). A pre-incubation time of 5 min was the minimum time required for optimal inhibition with 1.0 µM Lalistat 2.

Linearity of the method was assessed by incubating for up to 5 h and was shown to be adequate up to 3 h. Within-run coefficient of variation

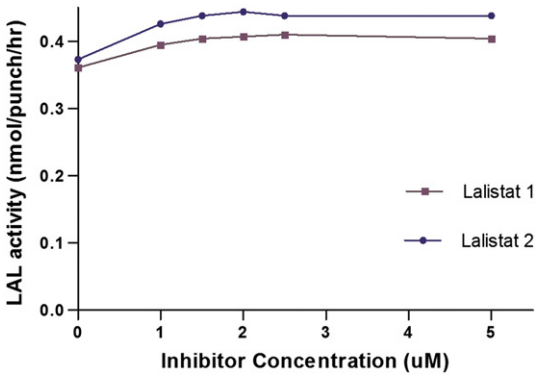

Fig. 3. Activity derived for LAL with Lalistat 1 and Lalistat 2 using pooled DBS extract.