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Optimization of ultra-high pressure liquid chromatography – tandem mass spectrometry determination in plasma and red blood cells of four sphingolipids and their evaluation as biomarker candidates of Gaucher's disease☆

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ABSTRACT

While important advances have been recently achieved in the optimization of lipid classes' separation, information on the specific determination of medium polarity lipids such as sphingolipids (SLs) in highly complex matrices remains fragmentary.

In human, disorders of SL metabolism known as sphingolipidoses are a heterogeneous group of inherited disorders affecting primarily the central nervous. Early diagnosis of these conditions is of importance notably when a corrective therapy is available. The diagnosis is generally based on the determination of specific SLs in plasma and red blood cells (RBCs). For instance, glucosylceramide (GL1), glucosylsphingosine (Lyso-GL1), sphingosine (Sph), and sphingosine-1-phosphate (S1P) are proposed as relevant biomarkers for Gaucher disease (GD).

Our main objective was to evaluate these biomarker candidates in a cohort of GD patients. However, most of current methods of GL1, Lyso-GL1, Sph, and S1P determination in plasma of GD patients require at least two liquid chromatographic runs. On the other hand, except for GL1 nothing is known concerning the RBC sphingolipid content. Yet, several reversed phase LC–MS methods of SLs separation and/or determination in various media with different sample preparation approaches have been proposed since 2010.

Here we focused on stationary phase selection and mobile phase composition as well as on the sample preparation step to optimize and validate an UHPLC–MS/MS method for the simultaneous quantification of the four sphingolipids in both plasma and RBCs. A comparison between seven stationary phases including two RP18, two polar embedded RP18, and three HILIC phases shows that under our conditions polar embedded RP18 phases are the most appropriate for the separation of the four SLs, in terms of efficiency, peak symmetry, and separation time. In the same way, a comparison between a single step extraction with methanol and a liquid–liquid extraction with a mixture of methanol/methyl *tert*-butyl ether, shows that the latter mixture is the most appropriate for the extraction of SLs in terms of recovery and absence of matrix effect. After validation, this method was applied to the evaluation of the targeted SLs in a cohort of 15 known GD patients. The obtained results show that Lyso-GL1 is the only relevant biomarker in both plasma and RBCs for GD diagnosis.

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As the proposed method is applicable to the determination in such a highly complex matrices of four SLs with a large difference in polarity, and as the sample preparation procedure is freedom of matrix effects, this method can be easily adapted to a large diversity of samples.

1. Introduction

Sphingolipids (SLs) are a class of lipids playing important roles in living organisms. Besides their structural role in cellular membranes, SLs are also involved in signal transmission and cell recognition thus playing essential functions in the regulation of cell growth, differentiation, senescence, and apoptosis [1]. In human, disorders of SL metabolism known as sphingolipidoses are a heterogeneous group of inherited disorders affecting primarily the central nervous system as reflected in Gaucher disease (GD), Fabry disease, Niemann-Pick disease, Krabbe disease, Tay-Sachs disease and Metachromatic leukodystrophy [2].

Early diagnosis of these disorders, which is generally based on the determination of specific SLs is of importance notably when a corrective therapy is available. This is the case for GD Type 1 (GD1, the non-neuronopathic form) where an enzyme replacement therapy is proposed [3–5] and where glucosylceramide (GL1), glucosylsphingosine (Lyso-GL1), sphingosine (Sph), and sphingosine-1-phosphate (S1P) (Fig. 1) are considered as relevant biomarkers [3,6,7].

GD is due to mutations in the GBA gene encoding the enzyme β-glucocerebrosidase (GBA) resulting in its deficiency. In healthy subjects GBA hydrolyzes GL1 into ceramide Cer and glucose (Fig. 1). The enzymatic deficiency thus leads to the accumulation of GL1 and its deacylated form Lyso-GL1 within the lysosomes [8]. In plasma the extra-lysosomal glucocerebrosidase GBA2 converts GL1 and Lyso-GL1 into ceramide (Cer) and sphingosine (Sph), respectively. Meanwhile, sphingosine kinase catalyzes the phosphorylation of the resulting sphingosine leading to the accumulation of sphingosine-1-phosphate (S1P) [7]. Thus, the lipids downstream of GBA2 namely Sph and S1P seem to be relevant biomarkers and GBA2 could potentially be targeted to ameliorate certain disabling manifestations of non-neuronopathic GD [3,7].

Standard diagnosis procedures and monitoring of GD patients include measurement of enzyme activity, genetic testing, and analysis of standard biomarkers of Gaucher cells like chitotriosidase and CCL18 [9]. However, these latter are not completely specific of GD and reliable biomarkers are still needed for evaluating the proposed enzyme replacement therapies as well as potential new therapeutics.

Initially our main objective was to evaluate these biomarker candidates in a cohort of GD patients. However, while important advances have been recently achieved in the optimization of lipid classes' separation [10–12] opinions remain divided on the specific separation of some classes such as SLs. Current methods of GL1, Lyso-GL1, Sph, and S1P determination in plasma require at least two liquid chromatographic runs. On the other hand, except for GL1 nothing is known concerning the RBC sphingolipid content [3,13,14].

Yet, several LC–MS methods either in RP or HILIC modes of SLs separation and/or determination in various media with different sample preparation approaches have been proposed since 2010 [10,15–21]. In 2010, HILIC separation coupled to MS detection was applied to quantify several SL species in cultured fibroblasts in less than 5 min [15]. In 2014 always in HILIC mode Sph, and S1P have been quantified in plasma after a one step methanol precipitation of proteins as sample preparation step on a zwitterionic stationary phase [18]. In 2015 a fast RP-UHPLC–MS/MS method for the simultaneous determination of 25 key SLs in human plasma has been proposed [19]. Although the gradient procedure is rather complex the separation of all targeted SLs have been achieved in less than 6 min with a sample preparation step consisting of a liquid–liquid extraction of total lipids with a mixture of chloroform/methanol [19]. One year later, an RP-HPLC–MS/MS method using a fused core Si-C18 column for the simultaneous determination in less than 20 min of 17 individual SL species in B cells collected from Chronic Lymphocytic Leukemia patients has been described [20]. Very recently, a C8-RP-UHPLC method has been proposed for the quantification of 11 SLs in plasma and 3 blood fractions (platelet-poor plasma, platelets, and RBCs) of women with mild preeclampsia [21]. A binary gradient was performed using ammonium formate and methanol. However, the chromatographic profile and retention times of SLs are not given [21].

Hence we focused on stationary phase selection and mobile phase composition as well as on the sample preparation step to develop and validate an UHPLC–MS/MS method for the simultaneous quantification of the four SLs in both plasma and RBCs. Seven stationary phases including two RP18 column (porous and fused-core particles), two polar embedded RP18 columns (3.0 μm and sub–2 μm particle sizes), and three HILIC stationary phases with different chemistries. The main objective was to optimize the separation in terms of efficiency, peak symmetry, and speed of separation. Also, for optimum robustness special attention was given for selecting the simplest gradient of elution and mobile phase composition. After validation, the proposed method was applied to the evaluation of the SL biomarker candidates in a cohort of 15 known GD patients.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, formic acid, and *tert*-butyl methyl ether (MTBE) were purchased from Carlo Erba (Val de Reuil, France). Ammonium formate was purchased from Sigma-Aldrich (St Quentin Fallavier, France). D-glucosyl-β1-1′-*N*-tetracosanoyl-D-*erythro*-sphingosine (GL1; d18:1/24:0) from Matreya LLC (PA, USA). D-glucosyl-β1-1′-D-*erythro*-sphingosine (Lyso-GL1; d18:1), D-erythro-sphingosine (Sph; d18:1), D-erythro-sphingosine 1-phosphate (S1P; d18:1), and the internal standards (IS) C17-D-*erythro*-sphingosine (C17B; d17:1), C17-D-*erythro*-sphingosine-1-phosphate (C17-S1P; d17:1), and *N*-heptadecanoyl-D-*erythro*-sphingosine (C17Cer; d18:1/17:0) were obtained from Avanti Polar Lipids (AL, USA).

2.2. UHPLC–MS/MS analysis

UHPLC–MS/MS experiments were performed on an Ultimate 3000 UHPLC system coupled to a TSQ Quantum Ultra mass spectrometer equipped with an ESI ion source. (Thermo Scientific, Courtaboeuf, France).

MS detection was operated in the positive mode with the following configuration: ion spray voltage, 4 kV; vaporizer temperature, 300 °C; capillary temperature, 300 °C; Capillary voltage 35 V; Tube lens and skimmer voltages were set at different values depend-

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