Clinica Chimica Acta 435 (2014) 1–6

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Preanalytical standardization of sphingosine-1-phosphate, sphinganine-1-phosphate and sphingosine analysis in human plasma by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 18 March 2014
Received in revised form 8 April 2014
Accepted 8 April 2014
Available online 24 April 2014

Keywords:
Sphingolipids
Human plasma
HILIC
Tandem mass spectrometry
Preanalytical standardization

ABSTRACT

Background: Preanalytical standardization is required for a reliable quantification of the signaling molecules sphingosine-1-phosphate (S1P), sphinganine-1-phosphate (SA1P) and sphingosine (SPH).
Methods: Methanolic protein precipitation of 15 μL EDTA-plasma was applied prior to analysis. Sphingolipids were separated in 3 min by hydrophilic interaction liquid chromatography (HILIC, SeQuant™ ZIC®-HILIC column) followed by tandem mass spectrometry. Stability of analytes in whole blood and plasma was investigated. Sphingolipid concentrations were determined in human plasma (n = 50) and mice deficient in sphingosine kinase 1 (SK1) and 2 (SK2) (n = 5).
Results: Storing EDTA whole blood >60 min after blood withdrawal at room temperature resulted in an increase in S1P and SPH concentrations of ≥25%. Significant changes in SPH levels of +37% were observed after 60 min of storage of EDTA plasma at room temperature. Repeated freeze–thaw cycles of EDTA plasma resulted in increased S1P and SPH levels. Concentrations in human EDTA plasma were between 55.5 and 145.2 ng/mL for S1P and between 8.9 and 35.3 ng/mL for SA1P. Concentrations of S1P were 36% lower and 96% higher in EDTA-plasma from SK1- and SK2-deficient mice, respectively, compared to the wild type.
Conclusions: Preanalytical standardization is a precondition for the analysis of sphingolipids in human blood.

1. Introduction

Sphingolipids such as sphingosine (SPH) and sphingosine-1-phosphate (S1P) are bioactive compounds that regulate cellular responses including proliferation, differentiation, migration and apoptosis [1–4]. However, S1P also functions as ligand for the G-protein coupled receptors $S1P_1$–$S1P_5$, which are expressed in cells of the immune, cardiovascular and nervous systems [5,6]. Extracellular S1P is released from activated platelets and is, therefore, an endogenous constituent of human plasma [7].

Sphingolipids are promising biomarkers for the diagnosis and therapy of diseases like diabetes, coronary heart disease and cancer [4,8–13]. In recent years, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has become a powerful tool in the analysis of sphingolipids [1–3,6,14–16]. However, laborious liquid–liquid extraction and/or derivatization steps as well as time-consuming LC-separations are the disadvantages of these methods that hinder an application in large-scale clinical studies. The application of hydrophilic interaction liquid chromatography (HILIC) enables the coelution of the sphingolipid species and their internal standards C17-S1P and C17-SPH which have the same polarity as the analytes. With this strategy, potential matrix effects or varying ionization efficiencies due to mobile phase gradients were compensated.

We developed a novel rapid LC–MS/MS method for the simultaneous quantification of sphingoid bases in human plasma including, sphingosine-1-phosphate, sphinganine-1-phosphate (SA1P) and sphingosine. We further defined a standardized preanalytical protocol for a reliable quantification of the analytes in human blood.

Abbreviations: C17-S1P, C17-sphingosine-1-phosphate; C17-SPH, C17-sphingosine; CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; IS, internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; LOD, limit of detection; MRM, multiple reaction monitoring; S/N, signal-to-noise ratio; S1P, sphingosine-1-phosphate; SA1P, sphinganine-1-phosphate; SK, sphingosine kinase; SPH, sphingosine; TCL, total change limit.

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http://dx.doi.org/10.1016/j.cca.2014.04.010
0009-8981/

2. Materials and methods

2.1. Subjects, animals, and sample collection

For method evaluation and stability experiments, whole blood of five donators was collected. The samples were centrifuged at 3200 ×*g* for 10 min.

EDTA plasma from 50 fasting apparently healthy volunteers (28 male/22 female, mean age 59.6 years) was collected to establish normal values. The samples were frozen after 10 min centrifugation at 3220 ×*g* and stored at −80 °C until analysis. The written informed consent was obtained from every individual included in the study, which was approved by the ethics committee of the University Leipzig (082-10-190-42010).

Mice deficient in sphingosine kinase 1 (SK1, n = 5) and 2 (SK2, n = 7) were generously donated by Richard Proia, NIDDK, Bethesda, USA and were described previously [17,18]. Blood samples from mice deficient in sphingosine kinase 1 (SK1) and 2 (SK2) [17,18] and wild-type mice (n = 8) were obtained from anesthetized animals by retroorbital puncture and immediately centrifuged after blood taking, and EDTA plasmas were stored at −80 °C until analysis.

2.2. Reagents and materials

ULC/MS grade acetonitrile, methanol, isopropanol and formic acid were purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid 37% (Merck, Darmstadt, Germany), ammonium formate (Fluka, Buchs, Switzerland) and dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) were of highest analytical grade available. Ultra-pure water was from a Barnstead NANOpure water purification system (Thermo Fisher Scientific, Waltham, MA, USA). SPH, S1P, SA1P and the internal standards C17-sphingosine (C17-SPH) and C17-sphingosine-1-phosphate (C17-S1P) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

2.3. Preparation of stock and working standards, quality control samples

Stock solutions of the lipid standards at a concentration of 1 g/L were prepared either in dimethyl sulfoxide/hydrochloric acid 32% (100:2, v/v) for S1P, SA1P and C17-S1P or in methanol/isopropanol (1:1, v/v) for SPH and C17-SPH. The stock solutions were stored at −20 °C and were further diluted with methanol to obtain working standards.

Four in-house quality control samples were used in each analytical batch. A methanolic quality control sample (with SPH, S1P, and SA1P levels of 10 ng/mL, 100 ng/mL and 25 ng/mL), a native and two spiked quality control samples (with SPH: 3 ng/mL and 6 ng/mL, with S1P: 175 ng/mL 300 ng/mL and with SA1P: 75 ng/mL and 150 ng/mL) were prepared.

2.4. LC–MS/MS analysis

The HPLC equipment consisted of two Series 200 Micro Pumps, a Series 200 Column Oven and a Series 200 Autosampler (Perkin Elmer, Waltham, USA). An API 4000™ LC/MS/MS system equipped with a Turbo V™ ion spray source operating in positive ESI mode was used for detection (Applied Biosystems, Darmstadt, Germany). The Turbo V™ ion spray source was operated using the following settings: ion spray voltage = 1500 V, ion source heater temperature = 300 °C, source gas 1 = 40 psi, source gas 2 = 50 psi, and curtain gas = 20 psi. The analytes were quantified using multiple reaction monitoring (MRM). Mass spectrometric parameters are summarized in Supplemental Table S1. Data analysis was performed with MultiQuant™ 2.0 (AB Sciex, Toronto, ON, Canada).

The chromatographic separation was performed on a SeQuant™ (Merck, Darmstadt, Germany) ZIC®-HILIC column (50 mm × 2.1 mm, 3.5 μm particle size). The column was maintained at 50 °C and the injection volume was 5 μL. The flow rate was set to 500 μL/min. The mobile phase consisted of 50 mmol/L ammonium formate in water/formic acid (100/0.2, v/v) as eluent A and acetonitrile/eluent A/formic acid (95/5/0.2, v/v/v) as eluent B. Gradient elution was performed with 0% A for 1.0 min, a linear increase to 50% A until 1.9 min, 50% A until 4.0 min and reequilibration from 4.1 to 6.0 min with 0% A.

2.5. Sample preparation

A modified single-step methanol extraction procedure according to Lan et al. was used [1]. In brief, 15 μL of human plasma and 85 μL of an internal standard solution containing 11.8 ng/mL C17-SPH and 588 ng/mL C17-S1P in methanol were mixed to yield final concentrations of 10 ng/mL and 500 ng/mL, respectively. The internal standard solution was stored at −50 °C. The mixture was vortex-mixed and centrifuged at 12,000 ×*g* for 5 min. The supernatant was transferred into glass vials prior to injection into the LC–ESI–MS/MS system.

2.6. Method evaluation

For calibration, ratios of analyte area and area of the internal standard were plotted against the analyte concentration. A signal-to-noise ratio (S/N) of 3 and 10 was used to calculate the limit of detection (LOD) and the lower limit of quantification (LLOQ) (n = 3). Linearity of S1P, SA1P and SPH was tested up to a concentration of 600 ng/mL, 200 ng/mL and 50 ng/mL, respectively.

Precision and recovery were assessed using our in-house prepared quality control samples. The within-day imprecision was determined by analyzing each quality control sample in one batch for 10 times. Between-day imprecision was calculated by measuring each quality control sample on 10 consecutive working days. Recovery was calculated by performing standard addition experiments with spiked plasma levels.

2.7. Stability of sphingoid bases

To investigate the stability of S1P, SA1P and SPH in human EDTA whole blood, freshly collected samples (n = 4) were aliquoted and stored at room temperature or 4 °C. Centrifugation of each individually stored whole blood aliquot was performed after 30 min, 60 min, 90 min and 120 min.

Analyte stability was investigated by the storage of pooled EDTA plasma at room temperature and at 4 °C for 0, 30, 60, 90 and 120 min, prior to sample preparation. The influence of five repeated freeze–thaw cycles on EDTA plasma (n = 5) was investigated. Therefore, samples were stored at −80 °C and refrozen on 5 consecutive working days. After each freeze–thaw cycle, sample preparation and LC–MS/MS analysis was performed.

Long-term stability was studied for one year using different storage tubes (polypropylene safe-lock tubes [Sarstedt, Nümbrecht, Germany], straws [Cryobiosystems, Paris, France] and cryotubes [Fluidx, Oakville, ON, Canada]) at −80 °C and −130 °C.

Stability of processed samples in the autosampler at room temperature was analyzed by repeated measurements of one pooled EDTA plasma sample for 11 h. Furthermore, the influence of up to 5 freeze–thaw cycles on a processed pooled EDTA plasma sample was investigated. Therefore, the sample was refrozen at −80 °C after each LC–MS/MS measurement.

2.8. Statistics

For statistical analysis, we used Microsoft Excel 2010 and IBM SPSS Statistics (version 20).

For the determination of normal concentrations, levels of SPH, S1P and SA1P were checked for normal distribution according to the Q–Q-Diagram. Gender specific concentration differences were calculated by the use of the Student's T-test, where p-values <0.05 were stated as