Preanalytical standardization is required for a reliable quantification of the signaling molecules sphingosine-1-phosphate (S1P), sphinganine-1-phosphate (SA1P) and sphingosine (SPH). 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.

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2. Materials and methods

2.1. Subjects, animals, and sample collection

For method evaluation and stability experiments, whole blood of five donors 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 B/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
statistically significant. Normal values were defined as mean ± 2 standard deviations.

Long-term stability was tested according to ISO 5725-6 [19]. In brief, the mean percentage difference was calculated according to the formula

\[ \frac{(T_x - T_0)}{T_0} \times 100, \]

where \( T_x \) and \( T_0 \) represent concentration values measured after a given time period and at the initial time point, respectively. We further calculated the total change limit (TCL). If the mean percentage difference of an analyte exceeded the TCL, the difference was considered to be significant.

3. Results

3.1. Validation of the analytical method

The application of hydrophilic interaction chromatography enabled an efficient chromatographic separation of SPH and the phosphorylated species S1P and SA1P in 2.8 min following a re-equilibration time of 3.2 min until the next analysis. A chromatogram of an EDTA plasma sample with a total run time of 6 min is presented in Fig. S1. LOD and LLOQ were found to be 3.0 ng/mL and 9.8 ng/mL, respectively, for S1P and SA1P whereas limits of 0.5 ng/mL and 1.7 ng/mL were determined for SPH. The standard curves of 5 methanolic calibrators were linear in the concentration ranges indicated in Table S2 with Pearson coefficients of correlation >0.999. As shown in Table S3, within-day imprecision and between-day imprecision were <12% for the phosphorylated species S1P and SA1P whereas coefficients of variation (CV) were <23% for SPH. Accuracy, assessed by standard addition experiments in EDTA plasma, ranged between 94.9% and 120.8% (Table S4).

3.2. Preanalytical influences on sphingolipid analysis

Compared to EDTA plasma, S1P levels were lower in citrated (−16%) and higher in lithium heparin plasma (+5%). However, the comparison of citrated and lithium heparin plasma showed a significant difference of 26% in determined S1P concentrations. Levels of SA1P and SPH did not differ between the investigated plasma matrices (Fig. 1).

In EDTA whole blood, stability analysis revealed a first significant increase in S1P and SPH concentrations of 33% and 25% after 90 min storage at room temperature after blood withdrawal. SA1P concentrations remained stable until 90 min and increased by 22% after 120 min storage at room temperature. However, storage of EDTA whole blood samples at 4 °C until centrifugation showed stability at least up to 120 min for all analytes.

The same applies to the short-term storage of EDTA plasma samples at 4 °C prior to sample preparation (Fig. 2). At room temperature, SPH levels in EDTA plasma increased after 30 min storage whereas S1P and SA1P levels remained stable for at least 120 min.

In EDTA plasma samples, which have undergone up to five freeze-thaw cycles prior to sample preparation, SA1P levels remained stable while S1P and SPH concentrations changed by −25% and +43% after the fourth and first freeze-thaw cycles, respectively (Fig. 3). The investigation of EDTA plasma, which was stored in safe-lock tubes up to one year at −80 °C revealed a decrease of 34% in SA1P levels while SPH and S1P concentrations remained stable for the entire storage period. The trends in concentration changes during the long-term storage of EDTA plasma at −80 °C are shown in Table 1. No significant differences between storage conditions of −80 °C and −130 °C or the type of storage tube used were detected.

Repeated freeze-thaw cycles of processed samples did not show any significant change in concentration levels of the investigated analytes (Fig. S2). Processed samples also showed to be stable at room temperature for at least 11 h (data not shown).

3.3. Method validation in mice and sphingolipid concentrations in a human healthy population

For method validation, concentrations of sphingoid bases were determined in plasma obtained from wild-type mice as well as SK1- and SK2-deficient mice, which are characterized by decreased and elevated S1P levels, respectively [17,18]. The analysis of plasma from SK1-deficient mice revealed 34%–65% lower median levels of S1P, SA1P and SPH (156.1 ng/mL, 18.5 ng/mL, 8.1 ng/mL) in comparison to concentrations determined in wild-type mice (241.60 ng/mL S1P, 60.9 ng/mL SA1P, 15.6 ng/mL SPH). In contrast, median plasma levels of sphingoid bases seen in SK2-deficient mice (473.0 ng/mL...
Fig. 3. Analyte stability in EDTA plasma for SPH (A), S1P (B) and SA1P (C).
In this study we developed and validated a preanalytical protocol and analytical method to determine S1P and its related compounds SA1P and SPH in EDTA-plasma using hydrophilic interaction chromatography coupled to tandem mass spectrometry. In contrast to reversed phase separations, the usage of a zwitterionic stationary phase enabled the coelution of the analytes with their respective internal standards C17-SPH and C17-S1P whose alkyl chain is by one methylene group shorter than the one of the analytes. Besides variations in sample handling and analyte degradation also potential variations in ionization efficiencies as well as matrix effects can be corrected by the application of these internal standards in combination with hydrophilic interaction chromatography leading to a reliable quantification of sphingoid bases.

In terms of analysis time, the presented method is comparable to the recently published ones. High throughput analysis is enabled due to the combined simple one step methanolic protein precipitation with a total run time of 6 min [1,2].

Compared to previously reported methods, which require 25–1000 μL of plasma per single analysis, the sample volume could be reduced to only 15 μL. With respect to limited sample amounts in clinical and epidemiological studies and especially in animal experiments like mouse model studies, this improvement is of particular importance for the applicability of the presented method [1,3,6,14,20].

The validity of the analytical method could be confirmed with an intra- and interassay variability between 7.9 and 22.7% and analyte recovery rates of approximately 100%. The quantification limits and linear ranges were sufficient to quantify endogenous sphingolipid levels in human plasma although marginal higher limits of detection were determined compared to Lan et al. [1].

Serum is an improper specimen in sphingolipid analysis due to the release of S1P from activated platelets [21,22]. Therefore, only common plasma types were investigated. Our results show that plasma types in healthy volunteers. Investigations of sphingolipid stability after blood withdrawal revealed an increase in analyte concentrations in EDTA whole blood if stored at room temperature for at least 90 min prior to centrifugation [6,23]. Therefore, the storage of EDTA whole blood at 4 °C is recommended. The same applies to the storage of EDTA plasma specimens during sample pretreatment since in contrast to published studies, a significant increase in SPH levels of 37% could be already detected after 60 min storage at room temperature [3,16]. During long-term storage of EDTA plasma specimens, S1P and SPH levels remained stable while SA1P levels decreased by −34% after a storage time of one year, which has to be considered with respect to conducting clinical or epidemiological studies. However, this concentration change is independent from the storage temperature. For SPH, changes in concentration levels were already determined after the first freeze–thaw cycle of EDTA plasma, which is, indeed, in contrast to the findings by Schmidt et al. but shows that repeated freeze–thaw cycles should be avoided [3].

Processed samples were stable for at least 11 h at room temperature, which allows maximum batch sizes of 110 samples. Furthermore, processed samples were stable over 5 freeze–thaw cycles, which on one hand facilitates repeated measurements on different working days and on the other hand allows the temporal separation of sample preparation and LC–MS/MS analysis.

By the application of the presented resource-saving procedure we could show that median plasma levels of S1P in SK1-deficient mice are lower by 34% and 74% higher in SK2-deficient mice in comparison to wild-type mice. In normal human EDTA plasma, S1P, SA1P and SPH levels were determined in lower concentration ranges as previously reported which might be due to the fasting status of the subjects included in the study [3,6,14,24]. The influence of diet on levels of sphingoid bases has to be further investigated to standardize the quantification of these analytes.

With respect to the performed stability experiments, the following preanalytical protocol has to be considered regarding a reliable quantification of S1P, SA1P and SPH in human plasma:

- After blood withdrawal, store EDTA whole blood at 4 °C for a maximum of 120 min until centrifugation.
- Store fresh, unfrozen EDTA plasma on ice until sample preparation.
- 15 μL sample + 85 μL IS in methanol, vortex, and centrifuge at 12,000 × g for 5 min.
- Transfer supernatant into autosampler vial and keep frozen until analysis.

According to the obtained stability results in EDTA plasma, we advise to consider increasing SPH levels after the first freeze–thaw cycle and decreasing SA1P concentrations during long term storage at −80 °C when analyzing and evaluating study samples.

### 5. Conclusion

We developed a fast HILIC–MS/MS method for the specific and sensitive analysis of S1P, SA1P and SPH in 15 μL human plasma including a rapid and gentle sample preparation. A reliable quantification of the sphingoid bases in epidemiological and clinical studies can be ensured by the definition of standardized preanalytical protocol.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2014.04.010.

### Acknowledgment

This publication is supported by LIFE–Leipzig Research Center for Civilization Diseases (LIFE-005-C2), Universität Leipzig, LIFE is funded by means of the European Union, by the European Regional Development Fund (ERDF) and by means of the Free State of Saxony within the framework of the excellence initiative. The authors have declared no conflict of interest.
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