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A Facile Stable-Isotope Dilution Method for Determination of Sphingosine Phosphate Lyase Activity

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Abstract

A new technique for quantifying sphingosine phosphate lyase activity in biological samples is described. In this procedure, 2-hydrazinoquinoline is used to convert (2*E*)-hexadecenal into the corresponding hydrazone derivative to improve ionization efficiency and selectivity of detection. Combined utilization of liquid chromatographic separation and multiple reaction monitoring-mass spectrometry allows for simultaneous quantification of the substrate S1P and product (2*E*)-hexadecenal. Incorporation of (2*E*)-d₅-hexadecenal as an internal standard improves detection accuracy and precision. A simple one-step derivatization procedure eliminates the need for further extractions. Limits of quantification for (2*E*)-hexadecenal and sphingosine-1-phosphate are 100 and 50 fmol, respectively. The assay displays a wide dynamic detection range useful for detection of low basal sphingosine phosphate lyase activity in wild type cells, SPL-overexpressing cell lines, and wild type mouse tissues. Compared to current methods, the capacity for simultaneous detection of sphingosine-1-phosphate and (2*E*)-hexadecenal greatly improves the accuracy of results and shows excellent sensitivity and specificity for sphingosine phosphate lyase activity detection.

Keywords

Sphingosine-1-phosphate; (2*E*)-hexadecenal; 2-hydrazinoquinoline; sphingosine phosphate lyase; sphingolipids; and sphingosine phosphate lyase

1. Introduction

Sphingosine-1-phosphate (S1P) is a phosphorylated sphingoid base and the final common product of sphingolipid degradation. It is a bioactive lipid that circulates in blood and lymph and acts as a ligand for a family of five G protein-coupled receptors, through which it mediates many important signaling functions (Proia and Hla, 2015). S1P signaling influences cell proliferation, migration, differentiation and apoptosis. Its synthesis and

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signaling functions are essential for normal mammalian development and contribute to many aspects of postnatal life including vascular, cardiac and brain development, lymphocyte trafficking, innate immune cell functions, stem cell biology and tissue regeneration (Fyrst and Saba, 2010; Maceyka and Spiegel, 2014).

S1P is synthesized by phosphorylation of sphingosine, a process catalyzed by sphingosine kinases (Pitson, 2011). In the reverse reaction, S1P can be dephosphorylated by specific and nonspecific lipid phosphatases, regenerating sphingosine (Brindley and Pilquill, 2009). Alternatively, S1P can be irreversibly cleaved at the C2-3 carbon bond, generating two products, ethanolamine phosphate and (2*E*)-hexadecenal in a reaction catalyzed by sphingosine phosphate lyase (SPL) (Kumar and Saba, 2009). SPL can similarly degrade other naturally occurring phosphorylated sphingoid bases of various chain lengths and saturation, resulting in formation of ethanolamine phosphate and the corresponding aliphatic fatty aldehyde (Stoffel et al., 1974; 1969). The aldehyde is converted (most likely through the actions of FALDH) to a fatty acid, which can be either reutilized in sphingolipid or phospholipid biosynthesis or further converted to an alcohol, the latter which can be incorporated into ether glycerolipids (Kihara, 2014; Nakahara et al., 2012). The enzymatic step catalyzed by SPL is the only pathway by which endogenous or dietary sphingolipids can be degraded. Hence, loss of SPL activity results in accumulation of numerous bioactive sphingolipid intermediates including phosphorylated and unphosphorylated sphingoid bases and ceramides.

SPL is an intrinsic protein of the endoplasmic reticulum by virtue of an N-terminal transmembrane spanning region (Bourquin et al., 2010; Ikeda et al., 2004). Its active site faces the cytosol, where it gains access to its substrate and cofactor. The holoenzyme includes the loosely associated cofactor pyridoxal 5'-phosphate (vitamin B6), which likely forms a Schiff base with the amino group of the substrate (Van Veldhoven, 2000). Crystallization of yeast and bacterial SPL proteins have revealed that the yeast (and possibly human) enzyme likely functions as a dimer (Bourquin et al., 2010). Analysis of the amino acid sequence suggests the potential for various types of post-translational modifications including phosphorylation, nitrosylation, and glycosylation. However, we are unaware of any reports describing experimental verification of these predictions.

SPL performs an essential function in mammals, as mice lacking SPL exhibit various developmental anomalies and survive only about a month after birth (Schmahl et al., 2007; 2008). Additional studies have implicated roles for SPL in invertebrate development, stress responses, lipid homeostasis, mitogenesis, endocytosis, tissue regeneration, inflammation, oncogenesis, neurodegeneration, muscle regeneration and other processes (Bektas et al., 2010; Herr et al., 2003; Kumar et al., 2011b; Kumar and Saba, 2009; Li et al., 2000; Mendel et al., 2003). Various phenotypes associated with loss of SPL activity have been attributed to accumulation of the bioactive substrate, accumulation of other bioactive sphingolipid intermediates, immediate functions of SPL products, and the impact of SPL products on phospholipid metabolism (Bektas et al., 2010).

SPL has been indirectly implicated in several human diseases. For example, the aldehyde product of the reaction catalyzed by SPL may contribute to manifestations of Sjogren-

Larsson syndrome, a disease caused by mutations in FALDH that result in accumulation of fatty aldehydes (Nakahara et al., 2012). Alterations in SPL gene and protein expression have been observed in other disease states (Abu El-Asrar et al., 2014; Brizuela:2012ew; Ceccom et al., 2014; Oskouian et al., 2006; Testai et al., 2015). However, thus far no SPL mutation has been shown to play a direct role in the pathophysiology of a human disease. In contrast, pharmacological targeting of SPL is being explored as an immunomodulatory strategy for the treatment of autoimmune diseases (Bagdanoff et al., 2010).

The first reported assay for measurement of SPL enzyme activity involved monitoring the formation of the fatty aldehyde product and corresponding fatty acid and alcohol by thin layer chromatography using a radioactive dihydrosphingosine substrate labeled at carbon C4-5 (Stoffel et al., 1974; 1969). This method represents a sensitive assay that allows for quantitation of the fatty aldehyde and its fatty acid and alcohol metabolites. It is tedious, however, involves use of radioactivity, and is not flexible for analysis of SPL activity toward phosphorylated sphingoid bases of unusual structures, such as those found in model organisms. Other SPL assays relying on detection of the aldehyde product derived from fluorescent substrates or derivatized and measured by gas or liquid chromatography/mass spectrometry (LC/MS) have subsequently been developed (Bandhuvula et al., 2007; 2009; Bedia et al., 2009; Billich et al., 2013; Kashem et al., 2014; Lüth et al., 2012; Mezzar et al., 2014). Each method has benefits and drawbacks; the latter include inflexibility regarding substrate structure, limitations of sensitivity and specificity, and reliance on expensive, commercially available substrates.

Herein, we describe a novel method for quantifying SPL activity based on monitoring production of the fatty aldehyde by liquid chromatography/mass spectrometry (LC/MS) after derivatization with 2-hydrazinoquinoline (HQ), which forms hydrazone with (2*E*)-hexadecenal (Yuwei Lu, 2013). A simple one-step derivatization process and incorporation of (2*E*)-d₅-hexadecenal stable-isotope improves quantification accuracy and precision. Additionally, LC/MS separation allows simultaneous quantification of sphingosine-1-phosphate (S1P) during the assay. This assay has wider dynamic range detection of sphingosine phosphate lyase (SPL) activity than previous assays that utilize NBD- or BODIPY-labeled substrates (Bandhuvula et al., 2009; 2007).

2. Material and methods

2.1 Reagents

Internal standards (2*E*)-hexadecenal-(15,15,16,16,16-d₅) and C17: 1 *D*-erythro-sphingosine-1-phosphate (C17 S1P) and C18: 1 *D*-erythro-sphingosine-1-phosphate (S1P) were purchased from Avanti polar lipids. (2*E*)-hexadecenal was synthesized and provided to us by Dr. Robert Bittman. Truncated mammalian SPL (SPL 1-61) was provided by Echelon Inc. HQ was purchased from Alfa Aesar. All other reagents were of the highest purity grades and purchased from Sigma-Aldrich.

2.2. Animals

All animal care, husbandry, euthanasia and tissue harvest protocols used in this study were approved by our Institutional Animal Care and Use Committee. Sgpl1^{-/-} mice and wild

type littermate controls were originally obtained from Philip Soriano, Mount Sinai School of Medicine, New York, and have been described previously (Bektas et al., 2010). The mice were maintained on a mixed C57BL/6/129sv background by heterozygous matings, with pups genotyped at about 15 days after birth and used for experiments at about 18 days after birth, prior to normal weaning age because of their shortened life span. Littermate *Sgpl1*^{+/+} (wild-type) mice were used as controls.

2.2 Cell Culture

HEK293T and human fibroblast cell lines were grown in DMEM supplemented with 10% FBS plus penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% CO₂ incubator at 37° C. Forced SPL overexpression in HEK293T cells was performed as described (Bandhuvula et al., 2009; 2007). Briefly, HEK293T cells were transfected with a pcDNA3 plasmid containing the human SPL cDNA sequence by the calcium phosphate precipitation method (Kingston et al., 2003). Twenty four hours after transfection, cells were given fresh media and 48 hours later cells were harvested by scraping. Cell pellets were washed twice with PBS and stored in -80° C.

2.3 Preparation of crude cellular homogenate for SPL assay

Fibroblast cell pellets were resuspended in 0.5 ml lysis buffer and lysed on ice with a probe sonicator for 30 seconds. The lysis buffer consisted of 50 mM potassium phosphate buffer pH 7.4, 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.2 mM pyridoxal 5'-phosphate, 1 mM PMSF, 11% (v/v) glycerol, and (EDTA-free) Roche protease inhibitor cocktail tablet. Cell debris was removed by low speed centrifugation at 100 × g for 5 min at 4° C. Protein concentration was determined using the Bradford assay (Biorad), according to the company's standard protocol and quantified by bovine serum albumin (BSA) as the standard protein. Samples were stored at -80°C until use.

2.4 Preparation of mouse tissue homogenate for SPL assay

Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. A small section (1–2 cm) of small intestine was removed from SPL wild-type (WT) and SPL knockout (KO) mice. Tissues were flushed and washed with PBS and were homogenized with a hand-held dounce homogenizer in tissue lysis buffer. Tissue lysis buffer consisted of 5 mM MOPS, 1 mM DTT, 1 mM EDTA, 0.2 mM pyridoxal 5'-phosphate, 0.25 M sucrose, 10% glycerol with 1 mM PMSF, and Roche EDTA-free protease inhibitor cocktail solution. The homogenates were subjected to centrifugation at 1000 × g for 5 min at 4° C to clarify the extracts. Protein concentrations in clarified supernatants were quantified by the Bradford assay (Biorad). Samples were stored at -80°C until use.

2.5 Standard SPL assay condition

Stock solutions of S1P were prepared by dissolving 5 mg of S1P in 10 ml of HPLC-grade methanol:water (90:10, v/v) solution and were stored in glass vials at -80 °C until use. Prior to use, stock solutions were incubated at 37 °C for 30 min and placed in a water-bath sonicator for 10 min. Eight µl aliquots of sonicated stock S1P solution (10.5 nmol) were

placed into a 4 ml glass vial and dried under a constant stream of nitrogen. Dried S1P were suspended in 20 μ l of HPLC grade water containing 0.8% Triton-X 100 (v/v).

For SPL assay, 20 μ l of tissue or cellular protein extracts of desired protein amount, as a source of SPL protein, was mixed with 160 μ l of pre-warmed (37 °C) SPL assay buffer. SPL assay buffer consisted of 36mM potassium phosphate buffer pH 7.4, 0.08% Triton X-100, 0.6mM EDTA, 0.4mM pyridoxal 5'-phosphate, 3 mM DTT, 70mM sucrose, 5mM sodium orthovanadate (Na_3VO_4) and 5 mM sodium fluoride (NaF). Inclusions of phosphatase inhibitors Na_3VO_4 and NaF are required to prevent non-SPL mediated S1P loss. SPL reaction was initiated by adding 20 μ l of S1P solution (50 μ M final) and incubated for up to 1 hour. At specified time points a 10 μ l aliquot was taken and mixed with 100 μ l of derivatization buffer. Derivatization buffer consisted of 5 mM 2HQ, 10 μ M C17-S1P, and 10 μ M (2E)-d₅-hexadecenal prepared in HPLC-grade acetonitrile acidified with 5% perchloric acid (v/v). This mixture was incubated at 60 °C for 60 min. 2HQ-derivatized samples were transferred into glass vials and 2 μ l was injected for LC-MS/MS analysis.

As a blank, cellular and protein extracts were incubated without S1P substrates for the duration of assay and processed as described above.

2.6 Preparation of Calibration Curves

Stock calibration solutions (1 mM) of (2E)-hexadecenal, 2-(15,15,16,16,16-d₅)-hexadecenal, were prepared in methanol and stored in -80 °C until use. For S1P and C17-S1P, compounds were suspended in methanol at 1 mM concentration and probe-sonicated for 5 seconds at 22kHz (Fisher Scientific sonic dismembrator model 60). Solutions were subsequently diluted in methanol and stored in -80 °C until use.

Standard curves for S1P and hexadecenal were prepared by serial dilutions to obtain concentration ranges of 0.5 to 100 μ M. Ten μ l aliquots calibration standards were mixed with 100 μ l of derivatization solution containing 5 mM 2HQ, and internal standards (C17-S1P and d₅-hexadecenal; 10 μ M final) prepared in acetonitrile acidified with perchloric acid (5% v/v). Derivatization was carried out at 60°C for 60 min and 2 μ l was injected on to the column for LC-MS/MS analysis.

2.7 (2E)-Hexadecenal degradation rate and recovery efficiency determination

Protein extracts from HEK293T cells, SPL-overexpressing HEK293T cells (SPL++), primary skin fibroblasts, and mouse colon tissues from SPL WT and SPL KO mice were extracted as described above. To monitor the rates of product degradation in different matrices, (2E)-hexadecenal (20 nmol) was incubated in the absence or presence of 25 μ g of protein extracts dissolved in SPL assay buffer without S1P and incubated at 37 °C. Aliquots (10 μ l) were taken at 30 and 60 min and derivatized with HQ as described above. The recovery yield of (2E)-hexadecenal in the different sample matrices tested was calculated by dividing the concentration of (2E)-hexadecenal determined in protein samples by the concentration obtained from (2E)-hexadecenal spiked in PBS alone.

2.8 Instrumentation and settings

For analysis of target analytes, a 1290 ultra high pressure LC system coupled to Agilent 6490 Triple Quadrupole (QqQ) MS equipped with Agilent Jet Stream (AJS)-electrospray ionization interface was used. The instrument was operated by Mass Hunter Workstation software. Precursor and product ion selection and optimization of collision energies were performed manually by flow injection of singly derivatized or pure analytical standards. A Luna C18(2) column (50×3.5 mm, 3 μ m particle size; Phenomenex) maintained at 50 °C was used for chromatographic separation of the analytes. A binary gradient of mobile phase A (water:formic acid 99.9:0.1 v/v) and B (methanol:formic acid 99.9:0.1 v/v) was delivered at a constant flow rate of 0.3 ml/min. The total run time was 6 min. Initial gradient condition was maintained at 72% B and a linear gradient to 100% B within 4 min and was returned to baseline condition at 4.1 min to allow for ~ 2 min column re-equilibration.

Analysis was carried out using multiple reaction monitoring (MRM) mode. The general source settings in the positive ionization modes were as follows: gas temperature 200 °C; gas flow, 16 Lmin⁻¹; nebulizer 20 psi; sheath gas temperature 250 °C; sheath gas flow 11 L min⁻¹; capillary voltage 3000 V; and nozzle voltage; 0V. The fragmentor voltage of 380V and a dwell time of 200 ms were used for all mass transitions, and both Q1 and Q3 resolutions were set to nominal mass unit resolution. The MRM transitions used for HQ-(2*E*)-hexadecenal derivative detection were m/z 380.6 $\rightarrow$ 144.1 [collision energy (CE); 48 V] and m/z 380.6 $\rightarrow$ 117.1(CE 60V). (2*E*)-d₅-hexadecenal was detected with MRM transitions of m/z 385.6 $\rightarrow$ 144.1 (CE 48 V) and 385.6 $\rightarrow$ 117.1 (CE 60V). The transitions for S1P were m/z 380.5 $\rightarrow$ 264.2 (CE 12 V) and m/z 380.5 $\rightarrow$ 82 (CE 40 V). C17-S1P MRM transitions were m/z 366.5 $\rightarrow$ 250.3 (CE 12 V) and m/z 366.5 $\rightarrow$ 82 (CE 40 V).

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla California). One-way analysis of variance was used for statistical comparison of three or more independent groups whereas Student's t-test was used for computing significance of mean difference between two independent groups. Significance level was set at $p < 0.05$.

3. Results and Discussion

Fatty aldehydes like hexadecenal require derivatization for their sensitive detection because of their reactivity to biological matrices and poor ionization characteristics that impede their sensitive detection by MS. Hydrazine compounds represent a group of compounds that have a diazane moiety (N-N) and terminal hydrazinyl nitrogens that act as strong nucleophiles. Hydrazine compounds such as 2,4-dinitrophenylhydrazine (DNPH), semicarbazide and 2-diphenylacetyl-1,3-indandion-1-hydrazone (DAIH) have previously been used to efficiently derivatize aliphatic aldehydes for MS detection (Berdyshev et al., 2011; Lüth et al., 2012). In this study, HQ was used for (2*E*)-hexadecenal derivatization and quantification. In contrast to other hydrazine compounds, in the presence of catalysts HQ efficiently derivatizes ketones and carboxylic acid and thus affords flexibility for detecting wider coverage of other potentially relevant small molecule targets, such as pyridoxal 5'-phosphate

and organic acids (Yuwei Lu, 2013). Developing an efficient and simple assay based on HQ derivatization may allow expanded coverage for metabolite profiling in future assays.

3.1 Optimization of HQ derivatization reactions

Reactions between HQ and (2*E*)-hexadecenal are shown in Figure 1A. HQ derivatization chemistry proceeds by initial nucleophilic attack of the (2*E*)-hexadecenal carbonyl carbon by terminal hydrazinyl nitrogen followed by dehydration and formation of a C-N bond (Figure 1A). The predicted hydrazone derivative product with a molecular mass of 379 is shown in Figure 1A. As described previously (Berdyshev, 2011), hydrazine reactions with aldehydes in methanol solvent are favored in acidic conditions. Thus, in the first phase of optimization, the efficiency of reactions in the presence of 2 and 5% perchloric acid (HClO₄) were compared to the reaction in methanol alone. HClO₄ was chosen because it is an effective protein precipitating agent that can rapidly quench metabolic reactions and is compatible with metabolite extractions (Gowda and Raftery, 2014). Derivatization with 5% HClO₄ was found to be optimal in promoting complete conversion of hexadecenal to its hydrazone derivative (data not shown). The reaction was carried out at 60 °C for 1 hour, and this condition was based on previously published results (Yuwei Lu, 2013).

Mass spectra analysis of the product formed by HQ derivatization was performed follow electrospray positive ionization, and the result (shown in Figure 1B) shows a single major dominant [M+H]⁺ mass of *m/z* 380, as predicted by the reaction chemistry. In agreement with previous reports (Yuwei Lu, 2013), fragmentation of the parent mass yielded a characteristic hydrazone fragmentation pattern (Figure 1C). To maximize MRM sensitivity, CE and cone voltage (CV; 5 V) were optimized for precursor to product transitions of *m/z* 380 → 234 (CE = 20 V) for quantification and *m/z* 380 → 114 (CE 40 mV) as a qualifier transition.

S1P does not react with HQ. However, it is sensitively detected by MRM-MS analysis and thus chromatographic separation of these compounds would allow simultaneous determination of both substrate and aldehyde product. For detection of S1P, a precursor to product transition of *m/z* 380.5 → 264.2, generated by the loss of the phosphate group, was used for quantification. C17 S1P, which is not endogenously synthesized, was used as an internal standard for S1P.

3.2 LC optimization

For improved selectivity and sensitivity, chromatography was optimized for resolving the 2HQ-(2*E*)-hexadecenal derivative from S1P. To achieve efficient separation, Luna C18(2) column (50 × 3.5 mm, 3 μm particle size) was used. Chromatographic resolution obtained by using acetonitrile and 0.1% ammonium formate was compared to that obtained by methanol and 0.1% formic acid. The peak asymmetry and resolution of S1P was found to be superior when methanol was used as the organic phase solvent (data not shown). Figure 2 shows the optimized separation of C17-S1P, (2*E*)-hexadecenal, (2*E*)-d₅-hexadecenal and S1P compounds. 2HQ-derivatives of (2*E*)-hexadecenal and d₅-hexadecenal eluted at 2.2 min. C17-S1P and S1P eluted at 2.6 and 3 min, respectively (Figure 2). Chromatographic profiles of (2*E*)-hexadecenal and (2*E*)-d₅-hexadecenal showed identical characteristics.

3.3 Recovery efficiency and linearity of response

To confirm efficient recovery of hexadecenal in different protein matrices, 10 μM hexadecenal was spiked into solutions containing 25 μg of boiled denatured proteins obtained from HEK239T, skin fibroblasts, and colon tissues, and the relative signals were compared to signal obtained without the protein incubations. Recovery across different matrices was excellent and showed greater than $84.5 \pm 4.9\%$ yield (data not shown) and was on par with other assays (Reina et al., 2012)

In order to facilitate accurate quantification that can account for potential matrix effects and derivatization yield, stable isotope-labeled internal standard (2*E*)-d₅-hexadecenal) was used. For simultaneous quantification of S1P, C17 S1P (which is absent in mammalian systems) was chosen as an internal standard. To determine the linearity of response and to establish detection limits of the assay, increasing amounts of S1P and HQ-derivatized (2*E*)-hexadecenal were injected, and the ratios of external signals and internal standard signal were plotted against concentrations injected (Figure 3A). Results showed excellent linear response over three orders of magnitude in dynamic range of detection. Based on our assay conditions, this translates into a dynamic range of 10 nM – 100 μM concentrations. The limits of detection (LOD; as defined by signal to noise ratio >2) and the limits of quantitation (LOQ) were determined to be 10 fmol and 100 fmol, respectively. The detection limit established in the current assay is similar to ones reported for using a semicarbazide derivatization assay (Berdyshev et al., 2011). The LOQ is lower than previously reported values of 287 fmol which was determined from perfluorobenzoyloxime-derivatized (2*E*)-hexadecenal (Reina et al., 2012). Linearity of response for S1P (shown in Figure 3B) also shows excellent linearity within the same concentration range as tested for (2*E*)-hexadecenal. S1P signals were ~10 fold higher than observed for (2*E*)-hexadecenal and showed correspondingly lower LOD of 5 fmol and quantification limits of 50 fmols (Figure 3B). These results show that both substrate and products of SPL can be sensitively quantified in a single injection analysis.

Simultaneous quantification of S1P and (2*E*)-hexadecenal allows this method to be easily adapted to measure steady-state concentrations of these compounds in cells and tissues. It should be noted that given poorer chromatographic resolution and potential carry-over artifacts with S1P, the quantification of SPL activity should primarily based on the rates of (2*E*)-hexadecenal formation.

To assess the linearity of activity measurements, the rates of (2*E*)-hexadecenal formation were determined with 0.5, 1, and 2 μg of commercially available N-terminal truncated SPL (1-61). The N-terminal region of SPL contains the luminal and transmembrane domains that are required for anchoring SPL to the endoplasmic reticulum membrane but non-essential for SPL activity (Van Veldhoven and Mannaerts, 1991) (Ikeda et al., 2004). Purified SPL was used to establish detection sensitivity of the SPL assay. SPL activity was initiated by addition of 100 μM S1P, and aliquots were taken at 15 and 30 min to assess the rates of S1P loss and (2*E*)-hexadecenal formation. As shown in Figure 4A, the current assay afforded sufficient sensitivity to detect activity of 0.1 μg of SPL (0.7 pmol/min) and was

linear to detect activities up to 2 μg of SPL. Protein-adjusted activity rates were similar across the three SPL concentrations tested, further supporting the linearity of response.

Steady-state enzyme kinetic parameters of recombinant SPL (0.5 μg) were also determined (Figure 4B and C) by monitoring the rates of (2*E*)-hexadecenal formation for 30 min. The Michaelis-Menten constant (K_m) and V_{max} were obtained by fitting the initial velocities obtained in the presence of 2, 3, 4, 6, 16, and 32 μM of S1P (Figure 4 B and C). Initial rates V (pmol/min/ μg protein) were calculated for each substrate concentration by calculating the rate of (2*E*)-hexadecenal formed during the first 30 min of the reaction. K_m values were determined by plotting the initial velocity against substrate concentrations (μM) and applying non-linear regression analysis based on the Michaelis-Menten equation (figure 4B) or by plotting a Lineweaver-Burk double reciprocal plots of $1/V$ against $1/S$ and using the linear regression analysis to calculate k_m and V_{max} (figure 4C). The K_m for S1P of the purified hSPL calculated using both approaches were similar; estimates based on Michaelis-Menten and Lineweaver-Burk plots were 4.67 ± 1.28 and 4.94 ± 0.32 μM , respectively (figure 4b and c). V_{max} estimates by both equations gave similar values of 2.6 pmol/min/ μg protein. Using S1P (Berdyshev et al., 2011) and C17-dihydroS1P (Reina et al., 2012) as substrates, K_m was previously estimated to be approximately 6 μM are similar to values that we obtained in this study. However, the calculated V_{max} in this study was significantly higher than previously published values of 0.17 pmol/min/ μg protein (Berdyshev et al., 2011) and 0.37 pmol/min/ μg protein (Reina et al., 2012). Several differences may contribute to this discrepancy. First, unlike the prior studies that used crude tissue or cellular extracts, kinetic assays were performed using purified hSPL. Secondly, we are using a recombinant human protein whereas previous studies were based on either mouse cell or tissue preparations. It is of note that SPL is a membrane enzyme and, thus, when assayed in purified state, may lack interactions with membrane lipids that modulate its activity. Further studies may be needed to determine the potential factors that modulate its kinetic parameters and to establish the physiological significance of these differences.

3.4 Detection of SPL activity in complex tissues

Fatty aldehydes that arise as part of normal metabolism such as (2*E*)-hexadecenal or as a result of lipid peroxidation are toxic due to their ability to form adducts to cellular macromolecules such as protein and nucleic acids (Kumar et al., 2011a; Upadhyaya et al., 2012). Several endogenous enzymatic detoxification enzymes, such as glutathione-s-transferase and aldehyde dehydrogenase, are present in tissues to efficiently neutralize the insidious effects of lipid aldehydes. It has been noted that (2*E*)-hexadecenal is principally detoxified by microsomal NAD^+ dependent fatty aldehyde dehydrogenase (FALDH) enzyme (Keller et al., 2014). Genetic mutations of FALDH cause Sjogren-Larsson Syndrome (SLS), a rare epithelial and neurological disorder. A previous study has shown that mouse liver microsomal protein preparations degrade (2*E*)-hexadecenal in a protein concentration-dependent manner (Berdyshev et al., 2011). However, the rates of (2*E*)-hexadecenal degradation in total cellular protein or tissue extracts from colon have not been previously tested.

To ascertain the endogenous rates of (2E)-hexadecenal degradation under our assay conditions, 20 nmol (2E)-hexadecenal was incubated in the presence of 25 µg cellular protein extracted from HEK239T cells, WT fibroblasts, and HEK293T cells over expressing SPL. This experiment was also performed in colon tissue extracts obtained from SPL-WT and KO mice. As shown in Figure 5A, endogenous rates of (2E)-hexadecenal degradation were similar to background rates of loss observed without any addition of protein. Although the rates appeared lower in fibroblast cell lines, these differences were not statistically significant. The rates of (2E)-hexadecenal degradation were determined in the presence of increasing concentrations of cellular protein obtained from HEK239T cells (Figure 5B). Results show similar rates of degradation across the different concentrations of protein extracts tested. These results suggest that, in contrast to microsomal protein preparations (Berdyshev et al., 2011), (2E)-hexadecenal degradation is minimal when whole cell or tissue extracts are used. This is likely due to the fact that FALDH is a microsomal enzyme and thus whole cell or tissue extract preparations may significantly dilute its effect on SPL assay.

As a reactive aldehyde, it has been suggested that (2E)-hexadecenal may react with S1P to form a Schiff base product (Bittman Laboratory, personal communications). To test this, (2E)-hexadecenal and S1P were mixed together with concentration ratios of 0.1, 1 and 10 under our assay conditions. Results showed no evidence of hexadecenal-S1P product formation (data not shown).

To determine the applicability of our assay in measuring SPL activity in cells, SPL activity was determined using 25, 50, 75 and 100 µg of cellular extracts obtained from HEK239T cells and 25 µg of protein obtained from HEK239T cells overexpressing SPL (SPL ++). As shown in Figure 5C, SPL activity increased linearly with increasing concentrations of added protein. Furthermore, almost 4000-fold higher activity was observed in whole cell extract of HEK239T cells transiently overexpressing SPL. This result clearly demonstrates the robust dynamic range of the current assay (Figure 5C). It is interesting to note that SPL activity in HEK239T cells stably transfected with pc-hSPL gene are significantly lower than the level of activity detected in transiently transfected HEK239T cells (Reiss et al., 2004). Thus, when comparing the cellular effects of SPL overexpression, it would be important to take this difference into account.

Lastly, to demonstrate the specificity of reaction, SPL activity was determined in 25 µg of colon tissue extract obtained from WT and KO mice (Figure 5D). Under the Vmax condition of SPL assay, an activity rate of 0.35 ± 0.05 pmol/min/µg was observed in WT tissue, and activity in the SPL KO tissue was below detection limits of the assay. The per µg activity of SPL activity in colon tissues are similar to Vmax rates published previously (Reina et al., 2012).

In summary, a simple and robust assay for measuring SPL activity in cells and tissues is described. Inclusion of both S1P and (2E)-hexadecenal detection in a single assay format provides confirmatory data to validate (2E)-hexadecenal results. Moreover, the stable isotope dilution technique used facilitates rapid quantification. The assay is sensitive enough to detect low basal activities in cells with limited SPL activity and has a robust dynamic range allowing the ability to distinguish high levels of activity in SPL- overexpressing cells.

Given the versatility of HQ in derivatizing ketones and carboxylic acids, this assay may be easily adapted for comprehensive polar metabolite detection and may be suitable for further development into a flexible metabolomics assay where steady-state concentrations of (2E)-hexadecenal can be measured simultaneously with other relevant metabolite targets.

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Abbreviations

HQ	2-hydrazinoquinoline
S1P	Sphingosine-1-phosphate
LC/MS	liquid chromatography/mass spectrometry
C17-S1P	sphingosine-1-phosphate
MRM	multiple reaction monitoring
CE	collision energy
CV	cone voltage
DNPH	2,4-dinitrophenylhydrazine
DAIH	2-diphenylacetyl-1,3-indandion-1-hydrazone

SPL sphingosine phosphate lyase

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Figure 1.

HQ efficiently derivatizes (2E)-hexadecenal through formation of hydrazones. Panel A. Reactions of carbonyl moiety of (2E)-hexadecenal with HQ yields a single hydrazone derivative with m/z of 380 $[M+H]^+$. Terminal hydrazinyl nitrogen in HQ is a strong nucleophile, which directly attacks the carbonyl carbon causing dehydration and subsequent formation of hydrazone. Panel B shows the mass spectra of products formed following HQ derivatization of (2E)-hexadecenal and shows a single dominant product at the predicted m/z . Panel C shows MS/MS spectra of the hydrazone derivative of (2E)-hexadecenal and shows a dominant fragment of m/z 144.1, consistent with hydrazone formation.

Figure 2. Representative chromatograms of HQ-derivatized (2E)-hexadecenal and (2E)-d₅-hexadecenal, and underivatized S1P and C17-S1P obtained through multiple reaction monitoring. The specific MRM transition pairs used for quantitation and the retention times for analytes detected are shown.

Figure 3.

Linearity of SPL assay detection of substrate and product. Linearity of responses are shown for (2*E*)-hexadecenal (panel A) and S1P (Panel B). Inset figures show the linearity of responses in the low pmol ranges. Both compounds show excellent linearity and wide-dynamic range of detection. Results are derived from averages of triplicate experiments.

Figure 4.

Characteristics of recombinant SPL using the SPL assay. Linearity of reaction and kinetic parameters of N-terminal truncated recombinant SPL (1-61) are shown. Panel A shows the rates of (2E)-hexadecenal formed in the presence of 0.5, 1 and 2 μg of SPL and 25 μg of HEK239 T cellular protein measured after 30 min of the reaction. Panel B showing the concentrations of S1P loss (black bar) and (2E)-hexadecenal formed (gray bar) reveals excellent uniformity. Panel C shows the steady-state kinetic parameters calculated by fitting the initial velocities obtained with 2, 5, 10, 50 and 100 μM S1P concentrations. Calculated K_m and V_{max} are indicated. Results are derived from averages of triplicate experiments.

Figure 5.

Determination of rates of (2E)-hexadecenal degradation and SPL activity in complex biological samples. Panel A shows the rates of (2E)-hexadecenal degradation in the absence or presence of 25 μg of cellular protein and tissue protein extract. Panel B shows the rate of (2E)-hexadecenal degradation in the absence or presence of 25, 50, 100 and 200 μg of cellular protein extract obtained from HEK239T cells. Panel C shows the SPL activity rate determined in 5, 25, 50, 75, or 100 μg of HEK239T cellular protein and in 25 μg of cellular protein extract obtained from SPL overexpressing HEK239T cells (SPL++). Panel D shows the SPL activity measured in WT and SPL KO mouse colon tissue protein extracts. Results are derived from the averages of triplicate experiments.