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A novel method to quantify sphingosine 1-phosphate by immobilized metal affinity chromatography (IMAC)

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Abstract

Sphingosine 1-phosphate (S1P), a lysophospholipid mediator that signals through G protein-coupled receptors, regulates a wide plethora of biological responses such as angiogenesis and immune cell trafficking. Detection and quantification of S1P in biological samples is challenging due to its unique physicochemical nature and occurrence in trace quantities. In this report, we describe a new method to selectively enrich S1P and dihydro-S1P from biological samples by the Fe^{3+} gel immobilized metal affinity chromatography (IMAC). The eluted S1P from IMAC was dephosphorylated, derivatized with *o*-phthalaldehyde (OPA), and detected by high-performance liquid chromatography (HPLC) coupled to a fluorescence detector. IMAC purification of S1P was linear for a wide range of S1P concentration. Using this assay, secretion of endogenous S1P from endothelial cells, fibroblasts and colon cancer cells was demonstrated. We also show that dihydro-S1P was the major sphingoid base phosphate secreted from HUVEC over expressed with *Sphk*1 cDNA. Pharmcological antagonists of ABC transporters, glyburide and MK-571 attenuated endogenous S1P release. This assay was also used to demonstrate that plasma S1P levels were not altered in mice deficient for ABC transporters, *Abca1*, *Abca7* and *Abcc1*/*Mrp1*. IMAC-based affinity-enrichment coupled with a HPLC-based separation and detection system is a rapid and sensitive method to accurately quantify S1P.

Keywords: Sphingosine 1-phosphate (S1P); Immobilized metal affinity chromatography (IMAC); Endothelial cells; Plasma S1P; Sphingosine kinase (*Sphk*); ABC transporters; High-performance liquid chromatography (HPLC)

1. Introduction

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid that regulates multiple biological processes. For example, S1P regulates angiogenesis [1,2], vascular permeability [3,4] vascular tone [5], heart rate [6], immune cell

Abbreviations: HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; IMAC, immobilized metal affinity chromatography; MEEC, mouse embryonic endothelial cells; MEF, mouse embryonic fibroblast; S1P, sphingosine 1-phosphate; S1Px, sphingosine 1-phosphate receptor X

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trafficking [7,8], inner ear development [9–11], mast cell function [12,13] and tumor growth [14,15]. Its actions are brought about by signaling through a family of G protein-coupled receptors termed as $S1P_1$–$S1P_5$ [16]. Despite the fact that S1P is a multifunctional mediator, the regulation of S1P synthesis and export from cells is poorly understood.

Mammalian blood contains high levels (∼1 μM) of S1P, which is bound to its chaperones—high-density lipoprotein (HDL) and albumin [17]. In contrast, tissue levels of S1P are low and therefore a concentration gradient of S1P exists between blood and interstitial fluids. However, mechanisms involved in the establishment and maintenance of plasma S1P gradient is not clearly understood. Recently, several groups suggested that erythrocytes might contribute significant amounts of S1P into blood plasma [18–20].

To determine the cellular sources of S1P and the mechanisms involved in the secretion of S1P from cultured cells, current methods utilize either [^{3}H]-sphingosine or inorganic [^{32}P]-phosphate labeling of cells followed by analysis of released S1P by TLC [21–23]. A potential weakness of these methodologies is that they do not directly measure endogenous S1P. In addition, classical methods require extraction of conditioned media with large volumes of solvents, and the presence of salt in the sample needs extensive washing steps. Although mass spectrometry-based methods are highly sensitive and quantitative, this methodology is not widely available due to the expense associated with instrumentation set-up, operational costs and the expertise required.

Here we describe a simple and rapid technique to enrich S1P from biological samples. Immobilized Fe^{3+} is known to bind tightly to phosphorylated amino acids and phosphopeptides [24]. This so-called immobilized metal affinity chromatography (IMAC) was adapted to selectively enrich S1P from other non-phospholipids. We show that IMAC affinity chromatography coupled with HPLC and fluorescence detection is capable of efficiently detecting small quantities of S1P from biological fluids and from conditioned media.

2. Materials and methods

2.1. Reagents and materials

S1P and C17-S1P were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid standards were dissolved in methanol and were stored at −20 °C. Glyburide, PHOS-Select™ iron affinity gel was purchased from Sigma (St. Louis, MO). MK-571 and Verapamil were purchased from Biomol (Plymouth, PA). [^{32}P]-ATP (specific activity 6000 Ci/mmol) was purchased from Amersham Life Sciences Products Inc. HPLC grade solvents; methanol, acetonitrile and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ). *Abca1*$^{+/-}$ breeding pair was purchased from Jackson Laboratory and bred in animal facility to derive *Abca1*$^{+/+}$, *Abca1*$^{+/-}$ and *Abca1*$^{-/-}$ animals. Plasma samples from *Abca7*$^{+/+}$ and *Abca7*$^{-/-}$ mice were provided by Dr. Michael L. Fitzgerald (Massachusetts General Hospital, Harvard Medical School, Boston, MA); while plasma and lymph from *Abcc1*$^{+/+}$ (also known as *Mrp1*) and *Abcc1*$^{-/-}$ mouse were provided by Dr. Gwendalyn J. Randolph (Mt. Sinai School of Medicine, New York, NY). Platelet poor plasma was isolated as described earlier [25].

2.2. Cell culture

Human umbilical vein vascular endothelial cells (HUVEC) were cultured as described [26]. Cell culture medium contained 10% fetal bovine serum (FBS), M-199, antibiotics and antimycotics, 5 units/ml of heparin and 150 μg/ml of endothelial cell growth supplement. While, mouse embryonic endothelial cells (MEEC), NIH3T3, mouse embryonic fibroblast (MEF); HCT-116, HT-29 cells were cultured in DMEM with 10% fetal bovine serum (FBS) having antibiotics and antimycotics in humidified CO_2 incubator.

2.3. S1P enrichment by IMAC

PHOS-Select™ iron affinity gel (40 μl of 50% beads slurry) was incubated with 2–5 ml of cell culture media, containing 5–10 pmol of internal standard C17-S1P. The binding of S1P with the IMAC resin was conducted in 250 mM acetic acid (pH 2.5–3.0) in 30% acetonitrile for 60 min at 4 °C. After washing the resin with 0.5 ml of 250 mM acetic acid (pH 2.5–3.0) in 30% acetonitrile and subsequently with 0.5 ml of deionized water, bound S1P was eluted in 0.5 ml of 150 or 400 mM ammonium hydroxide in 25% acetonitrile.