A peptide immunoaffinity LC–MS/MS strategy for quantifying the GPCR protein, S1PR1 in human colon biopsies

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Background: S1PR1, a G protein-coupled receptor (GPCR) protein, is a therapeutic target for treatment of autoimmune diseases. As a potential biomarker for drug effect and patient stratification, it is of great significance to measure it in biological samples. However, due to the hydrophobic nature of S1PR1 and the difficulties in extraction and solubilization, as well as low expression levels, quantitative determination of S1PR1 remains challenging. **Results:** In this work, a peptide immunoaffinity LC–MS/MS method was developed to quantify S1PR1 in biopsy-sized colon samples with an LLOQ of 7.81 pM. **Conclusion:** Peptide immunoaffinity LC–MS/MS based strategy has achieved the desired sensitivity for low abundance S1PR1, and the same strategy could be applied to quantify S1PR1 in multiple species and other GPCR proteins.

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Sphingosine-1-phosphate (S1P) is a lysophospholipid mediator that evokes a variety of cellular responses such as proliferation, migration, survival, angiogenesis, inflammation and lymphocyte egress by stimulation of five members of G-protein coupled receptors S1PR1, R2, R3, R4, R5, of which the S1PR1 receptor is most extensively investigated [1,2]. S1PR1 receptors are expressed in many cell types including lymphocytes and endothelial cells [3]. S1P induces sustained S1PR1 signaling with receptor recycling. Targeting S1P/ S1PR1 pathway has the potential to be an effective therapy for broad indications including lymphocyte-mediated autoimmune diseases [3–5]. Synthetic S1PR1 agonists induce transient S1PR1 signaling, internalization and degradation, and work as functional antagonists to inhibit S1P induced cell migration [4,6]. As a result, lymphocytes are sequestered in the thymus and secondary lymphoid organs without entering circulation. S1PR1 levels could be a potential biomarker for drug effect and patient stratification, and a quantitative assay suitable for colon biopsy samples was needed. To our knowledge, there have been no absolute quantitative assays for S1PR1 concentration levels in biological samples reported.

S1PR1 belongs to the family of G protein-coupled receptors (GPCRs), which are also known as seven-transmembrane domain receptors. GPCRs share common structure, with an extracellular N-terminus, 7-transmembrane helices linked by three extracellular (EL1–3) loops and three intracellular loops (IL1–3), and an intracellular C-terminus (Figure 1). The seven-transmembrane helices are arranged in a structurally conserved bundle and highly hydrophobic. GPCRs are the largest and most diverse group of membrane receptors with hundreds of different members. As cell–surface signal transmission receptors, they mediate most of the physiological responses to growth factors, hormones, neurotransmitters and environmental stimulants [7] and transduce an extracellular signal to an intracellular response through their transmembrane. Their importance is demonstrated by the fact that more than one-third of the currently marketed therapeutic medicines target more than 100 unique GPCRs for a broad spectrum of indications including cancer, infection, inflammation and cardiovascular disease etc. [8–10]. Furthermore, approximately 20% of new agents currently in clinical trials target more than 60 novel GPCRs [9]. Due to recent advances in structural biology, pharmacology and biotechnology, GPCR drug discovery

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Figure 1. Structural biology of the S1PR1 receptor.

has been growing fast, as demonstrated by numerous emerging novel drug targets. Bioanalytical strategies to measure GPCR proteins are of significant interest for drug discovery and development. However, due to their hydrophobic nature and intrinsic difficulties of extraction and solubilization, as well as low expression levels, direct quantitative determination of GPCR proteins remains challenging. The measurement of mRNA expression is usually used for prediction of relative expressions of GPCRs in cells and tissues [11]. For development of immuno-based assays, several obstacles have been encountered around the generation of specific antibodies against the GPCR proteins. First, detergent based solubilization of the GPCR immunogen causes major conformational changes but even subtle changes in conformation could affect the access of antibodies to the epitopes [12]. Second, since the epitopes are made up of small discontinuous extracellular segments, the chance for producing a strong immune response is significantly weakened. As a result, it is extremely difficult to generate specific and high-affinity antibodies. The dominant issue for GPCR antibody is lack of selectivity and a high level of cross-reactivity with other GPCRs due to the similarity in their protein sequences [13]. Third, it is difficult to obtain sufficient material for immunization due to low expression levels and solubility. It is also difficult to express them recombinantly. Given these difficulties, it is challenging to develop anti-protein based assays for GPCR protein quantitation.

In this work, our strategy is peptide immunoaffinity enrichment followed by LC–MS/MS detection. It bypasses most of those obstacles encountered for anti-protein immunoaffinity enrichment. Peptide immunoaffinity enrichment specifically captures the peptides of interest from complex biological matrices. Mass spectrometry has evolved as a powerful technology platform for specific detection of proteins from peptide level based on their mass-to-charge ratio and CID induced fragments [14–19]. Coupling peptide immunoaffinity enrichment with LC–MS/MS detection enables sufficient sensitivity and specificity for low-abundance endogenous proteins [20–26] and avoids the restrictions associated with traditional sandwich immunoassays. The LC–MS/MS approach could substantially save the time and cost involved in screening for a pair of antibodies [27,28]. Peptide immunoaffinity LC–MS/MS assays, also termed stable isotope standards and capture by anti-peptide antibodies (SISCAPA) as first reported by Anderson *et al.* [29], have been employed for protein biomarker quantitation as a complementary bioanalytical strategy to avoid the limitations inherent to immunoassays, such as potential interference from endogenous immunoglobulins [22]. Whiteaker *et al.* demonstrated the application of a multiplexed immuno-MRM assay targeting phospho-signaling [30,31]. In this work, we developed a peptide immunoaffinity LC–MS/MS assay for quantitative determination of a GPCR protein, S1PR1 in small biopsy-sized human colon tissue samples. The sample size was limited to a small-sized biopsy, since the ultimate goal was to apply this approach to clinical studies where only colon biopsy samples are collected by endoscopy. This paper details the challenges, strategies and method optimization for sample extraction, peptide selection and immunocapture to achieve the desired sensitivity and selectivity. Since all the GPCR proteins share similar structures, from bioanalytical perspective, it is very important to understand these challenges and establish bioanalytical strategies for assay development for GPCR protein quantitation.

Table 1. Multiple reaction monitoring transitions, declustering potential and collision energy for the surrogate peptides and the stable-isotope-labeled peptides.

Peptide	Precursor ion	Product ion	DP	CE
LTPAQWFLR.+2y7.light	566.3	917.5	72.4	28
LTPAQWFLR.+2y6.light	566.3	820.4	72.4	32
LTPAQWFLR.+2y7.heavy	571.3	927.5	72.4	28
LTPAQWFLR.+2y6.heavy	571.3	830.5	72.4	32
SSVSDYVNYDIIVR.+2y8.light	815.4	991.6	90.6	37
SSVSDYVNYDIIVR.+2y7.light	815.4	892.5	90.6	38
SSVSDYVNYDIIVR.+2y8.heavy	820.4	1001.6	90.6	37
SSVSDYVNYDIIVR.+2y7.heavy	820.4	902.5	90.6	38

CE: Collision energy; DP: Declustering potential.

Experimental

Materials & reagents

Recombinant human S1PR1 (H00001901-G01) containing 382 amino acids with molecular weight of 42.8 kDa was obtained from Abnova. It is a human S1PR1 full-length recombinant protein without tag. Anti-peptide antibodies, anti-LTPAQWFLR and anti-SSVSDYVNYDIIVR, which are rabbit polyclonal antibodies, were custom-made at GenScript. Magnetic tosylactivated beads (Invitrogen Dynabeads M-280), magnetic protein G beads (Invitrogen Dynabeads), Pierce DTT (Dithiothreitol) and Pierce iodoacetamide for single-use, HALT protease inhibitor cocktail and protein extraction detergents including T-PER, RIPA, CHAPS, NP-40, Triton X-100, Brij®-35, were obtained from Thermo Fisher Scientific (CA, USA). Trypsin Gold, sequencing grade-modified trypsin was obtained from Promega Corporation. Bovine serum albumin (BSA), phosphate buffered saline (PBS) and bovine pancreatic trypsin were purchased from Sigma-Aldrich. Stable-isotope-labeled (SIL) analogs of the surrogate peptides, SIL-LTPAQWFLR and SIL-SSVSDYVNYDIIVR, which were labeled with heavy arginine with a mass shift of +10, were custom-synthesized at New England Peptide. PBST buffer was prepared by adding 0.05% Tween-20 into PBS. Zwittergent 3–12 detergent was obtained from Emdmillipore. Human colon samples were purchased from Analytical Biological Services Inc.

Instrumentation & LC–MS/MS conditions

A quadrupole Orbitrap (Q Exactive, Thermo Fisher Scientific) operated in positive ESI mode was coupled to a LC system for surrogate peptide identification from recombinant human S1PR1 tryptic digest. Data-dependent acquisition (DDA) was performed to collect MS1 full scan and MS/MS spectra. Proteome Discoverer software was used to process data for peptide identification. Skyline was employed for building multiple reaction monitoring (MRM) acquisition methods for the collision energy (CE) optimization. AB SCIEX Triple Quad 6500 system (ABI/MDS Sciex, Concord, ON, Canada) equipped with a TurboIonspray source in positive MRM mode was used for detection of the surrogate peptides. Q1 and Q3 were operated in unit resolution. Quantitative analysis was conducted by using Analyst 1.6 software (AB Sciex). Two surrogate peptides, LTPAQWFLR and SSVSDYVNYDIIVR were identified and two MRM transitions were monitored for each peptide. The precursor and product ions of MRM transitions, their optimized collision energy and their default declustering potential predicted from Skyline, for the surrogate peptides and corresponding SIL peptides are listed in Table 1. A Waters Acquity UPLC I-Class system was coupled to the mass spectrometer for LC separation. The binary gradient was applied to a 2.1 × 50 mm, 1.8 µm ACQUITY™ UPLC™ HSS C18 column, which was maintained at 50°C, with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) mobile phases at a flow rate of 800 µl/min. The gradient started at 10% B, and held for 0.5 min. Then the gradient was ramped up linearly from 10% B to 35% B over 2.5 min followed by washing with 95% B and column equilibration. The chromatographic run time was 5 min.

Standard & quality control

The stock solution of S1PR1 recombinant protein has a concentration of 0.02 µg/µl in 25 mM Tris-HCl buffer of pH 8.0 containing 2% glycerol. It was diluted to 100 nM in PBS buffer containing 0.05% zwittergent and stored at -80°C. Calibration standards (STD) of S1PR1 recombinant protein at 7.81, 15.6, 31.3, 62.5, 125, 250 and

Table 2. The composition of extraction buffer.

Extraction buffer	Composition	Ionicity
PBS	Phosphates and sodium salts	Isotonic
T-PER	Proprietary detergent in 25 mM bicine, 150 mM NaCl	Zwitterionic
5% CHAPS in PBS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	Zwitterionic
RIPA	25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS	Nonionic/ anionic
5% NP-40 in PBS	10% NP-40 (nonyl phenoxyethoxyethanol) in Ultrapure Water Diluted 1:2 in PBS	Nonionic
1% Triton X-100 in PBS	10% Triton X-100 in Ultrapure Water Diluted 1:10 in PBS	Nonionic
5% Triton X-100 in PBS	10% Triton X-100 in Ultrapure Water Diluted 1:2 in PBS	Nonionic
5% Brij®-35 in PBS	10% Brij®-35 in Ultrapure Water Diluted 1:2 in PBS Contains ethoxylated lauryl alcohol	Nonionic

PBS: Phosphate buffered saline.

500 pM were prepared by serial dilution in lysis buffer containing 0.1% BSA and 1% HALT protease inhibitor cocktail. With the same stock solution, quality controls (QCs) of S1PR1 recombinant protein at 15.6, 31.3, 62.5, 125, 250 and 500 pM were prepared separately and three replicates for each level were analyzed.

The stock solution of SIL-LTPAQWFLR was prepared in 50 mM ammonium bicarbonate buffer containing 0.05% zwittergent at a concentration of 1 mM and stored at -80°C . The calibration standards of heavy surrogate peptide, SIL -LTPAQWFLR at 7.81, 15.6, 31.3, 62.5, 125, 250 and 500 pM were prepared by serial dilution in 50 mM ammonium bicarbonate buffer containing 0.05% zwittergent. With the same stock solution, QCs of heavy surrogate peptide, SIL -LTPAQWFLR at 15.6, 31.3, 62.5, 125, 250 and 500 pM were prepared separately and three replicates for each level were analyzed.

Tissue colon pulverization, homogenization & total protein measurement

Tissue homogenization

Colon tissue sample was pulverized into powder in TT1 tissue tube (Covaris, Inc.) by using CryoPREP™ Impactors (Covaris, Inc.) right after it was dipped in liquid nitrogen. Aliquots of the pulverized colon (~ 5 mg) were put into 0.5 ml CK14 homogenization tubes containing 1.4 mm ceramic (zirconium oxide) beads (Bertin Instruments). A volume of 200 μl of T-PER buffer with 1% HALT protease inhibitor cocktail was added to the samples. The samples were then homogenized at 4°C using two cycles of 30 sec at 6500 rpm with pauses of 30 sec between each cycle by using the Precellys Evolution Homogenizer with Cryolys Evolution (Bertin Instruments). Thus, total processing time for lysis is 2 min. Following centrifugation at $14,000 \times g$ for 10 min at 4°C , the supernatant was collected for immunocapture and total protein measurement. The total protein was measured by using a micro BCA protein assay kit (Thermo Fisher Scientific) following manufacturer's protocol. The measured concentration levels of S1PR1 were normalized to the total mg amount of protein in homogenate (pg/mg total protein) to enable direct comparison across samples.

Evaluation of the extraction efficiency of detergent lysis buffer

Several types of detergent lysis buffer were evaluated and compared for the extraction of S1PR1. PBS buffer was included as well for the purpose of comparison. The extraction buffer and its composition are shown in Table 2. Aliquots of the pulverized human colon were transferred into homogenization tubes in 2 replicates and homogenized in ten volumes of various extraction buffer containing 1% HALT protease inhibitor cocktail. Following centrifugation at $14,000 \times g$ for 10 min at 4°C , the supernatant was collected for tryptic digestion /immunocapture.

Preparations of Ab immobilized magnetic beads

Prior to sample preparation, anti-peptide antibodies, anti-LTPAQWFLR and anti-SSVSDYVNYDIIVR were covalently immobilized to magnetic tosylactivated beads, respectively. A suspension of 500 μl of magnetic beads was placed in a vial followed by washing 3 times with buffer A (0.1 M borate buffer, pH 9.5). After the third wash,

beads were combined with 303 μ l of 1 mg/ml antibody in PBS and 151.5 μ l of buffer C (3 M ammonium sulfate in buffer A). The mixture was incubated on a rotator at 37°C overnight. The beads were pelleted, and washed twice with 1 ml of glycine buffer (pH = 2.6) to remove potential interference (Ab reagents may contain trace level of antigens), and three-times with 1 ml of buffer D (PBS, pH 7.4 with 0.5% [w/v] bovine serum albumin). The beads were resuspended in 1 ml of buffer D and incubated on a rotator at 37°C for 1 h. After incubation, the beads were washed twice with 1 ml of buffer E (PBS, pH 7.4 with 0.1% [w/v] bovine serum albumin) and resuspended in 3 ml of buffer E. The beads were stored at 4°C.

The antibody immobilized protein G beads were prepared for comparison with tosylactivated beads. Anti-LTPAQWFLR and anti-SSVSDYVNYDIIVR antibodies were immobilized to magnetic protein G beads, respectively. A suspension of 1 ml of magnetic protein G beads was placed in a vial followed by washing 3 times with PBST buffer. The beads were resuspended in 1 ml of PBST buffer and combined with 320 μ g of antibody in PBS. The mixture was incubated on a rotator at RT for 2 h. After incubation, the beads were washed 3 times with 1 ml of PBST buffer containing 1% bovine serum albumin and resuspended in the same buffer. The beads were stored at 4°C.

Sample preparation: protein precipitation followed by pellet digestion & immunocapture

A volume of 100 μ l of colon tissue extract (supernatant after lysis) was transferred into a 96-well plate (Eppendorf 1 ml Protein LowBind), followed by addition of 400 μ l of methanol. The samples were vortex-mixed and shaken for 10 min. Following centrifugation at 2000 rpm for 10 min, the supernatants were carefully removed by a Perkin Elmer Janus automated liquid handler. The resulting pellets were resuspended in 500 μ l of 50 mM ammonium bicarbonate buffer containing 0.05% zwittergent by vigorous vortex-mixing for 15 min. The samples were then digested with 10 μ g of trypsin (Promega Trypsin Gold) overnight at 37°C. After digestion, the samples were incubated at 99°C for 30 min to deactivate trypsin. After the samples were cooled down to room temperature, they were combined with 100 μ l of PBST and 25 μ l of the antibody immobilized tosylactivated beads for each peptide, and incubated at RT for 1 h for anti-peptide immunocapture. The following washing and elution steps were carried out by KingFisher™ Flex System (Thermo Fisher Scientific). The beads were washed three times with 250 μ l of PBST buffer and one time with 250 μ l of 50 mM ammonium bicarbonate containing 0.05% zwittergent. The captured surrogate peptides were eluted from beads by using 100 μ l of 5% acetic acid containing 0.05% zwittergent. An aliquot of 40 μ l was injected for LC–MS/MS analysis.

Results & discussion

Anti-peptide strategy for S1PR1

Due to exceedingly low endogenous levels of S1PR1 and biopsy-sized colon tissue (~5 mg colon), a highly sensitive method was required. Immunoaffinity enrichment is the method of choice for sample cleanup to achieve the desired sensitivity. There are some commercially available antibody reagents for S1PR1. Some of these reagents have been tested in our lab for anti-protein immunoaffinity enrichment, but without success. Given the challenges discussed in the introduction section, anti-protein based immunoaffinity enrichment for GPCRs has had limited success. Our assay development strategy involves trypsin digestion followed by peptide immunoaffinity enrichment. The peptides of interest are specifically detected by LC–MS/MS. Rabbit polyclonal antibodies, which were raised against the surrogate peptide sequences, SSVSDYVNYDIIVR and LTPAQWFLR were custom-made for peptide immunoaffinity enrichment. The workflow is shown in Figure 2. Through denaturation, the tightly folded proteins are unfolded so that trypsin can easily access the proteolytic cleavage sites. Proteins are digested into complex mixture of tryptic peptides including the peptides of interest and the high level of peptides from matrix proteins. By immunoaffinity enrichment, the peptides of interest are isolated and the high level of matrix peptides are washed away. The peptides of interest are eluted from beads by acid and then detected by LC–MS/MS.

Selection of surrogate peptides

Figure 1 shows the structural biology of the S1PR1 receptor. Since seven transmembrane helices are structurally conserved and highly hydrophobic, usually the chance to detect the sequences at these regions by mass spectrometer is small. As a result, the overall sequence coverage is low and this leaves very limited choices for the selection of surrogate peptides. The surrogate peptides are mainly from the extracellular and intracellular segments. Two surrogate peptides, SSVSDYVNYDIIVR and LTPAQWFLR were identified by mass spectrometer. As shown in Figure 1, SSVSDYVNYDIIVR is located at extracellular N-terminus while LTPAQWFLR is mainly from first

Figure 2. The workflow of peptide immunoaffinity LC-MS/MS for S1PR1 quantitation.

extracellular loop. Table 1 shows MRM transitions and collision energy (CE) for the surrogate peptides and SIL peptides. Two MRM transitions were monitored for each peptide. Since the surrogate peptide LTPAQWFLR with MRM transition (566.3 > 917.5) provided desired sensitivity for 5 mg colon sample analysis, it was used in the final method for quantitation, while the other peptide and transitions were used for confirmatory purpose.

Extraction & solubilization of S1PR1 from colon tissue

Efficient extraction and solubilization of multi-transmembrane proteins from the phospholipid bilayer environment is critical for their quantitation [32]. However, due to their hydrophobic nature, multi-transmembrane proteins are usually difficult to extract and solubilize. Choice of detergents was carefully considered and their extraction efficiency was examined with respect to mass spectrometric response. As shown in Figure 1, seven helices of transmembrane protein pass through the biological membrane, and interact with the hydrophobic tails of the lipid molecules inside of the bilayer, while loops, N-terminus and C-terminus stay in the aqueous environment on either side of the membrane. Due to the similar amphiphilic nature, detergents are commonly used to extract transmembrane proteins by disrupting the phospholipid bilayers and solubilizing membrane proteins. We evaluated several commonly used detergents, including T-PER, 5% CHAPS, RIPA, 5% NP-40, Triton X-100 of 1 and 5%, and 5% Brij-35, for extraction of endogenous S1PR1 from human colon tissue. PBS buffer was included as well for the purpose of comparison. The extract buffer and its composition are listed in Table 2. The detailed sample preparation procedure was described in the experiment section. The results are summarized in Figure 3. An increase of Triton X-100 from 1 to 5%, as expected, leads to higher efficiency. The top four detergents are 5% NP-40, 5% Triton X-100, T-PER and RIPA.

Optimization of sample preparation

Considering that endogenous proteins are not identical to recombinant proteins, in this work, we used equal volume aliquots of cynomolgus monkey or human colon tissue homogenate to optimize the sample preparation procedures. Due to hydrophobic nature of S1PR1 and its surrogate peptide, LTPAQWFLR, significant loss of analytes was observed during sample processing. Surfactant, Zwittergent 3–12 detergent (0.05% in water) was added to stock and working solution, and the most of sample preparation steps to minimize the loss and enhance assay performance and sensitivity.

As shown in the workflow of Figure 2, the first step of sample preparation is protein denaturation. We have evaluated two types of denaturation methods, protein precipitation with methanol and heat denaturation at 99°C. For the protein precipitation approach, equal volume aliquots of human colon homogenate were precipitated with methanol at ratios of 1:2, 1:3 and 1:4, respectively. The supernatant was removed, and resulting pellets were

Figure 3. The comparison of LC-MS/MS response of endogenous S1PR1 in human colon tissue by using different extraction buffer. Please see Table 2 for the buffer composition. Peak area count of the surrogate peptide LTPAQWFLR, which is from endogenous S1PR1 in human colon tissue sample.

Figure 4. The effect of denaturation conditions on LC-MS/MS response of the surrogate peptide LTPAQWFLR, which is from endogenous S1PR1 in human colon homogenate. Protein precipitation denaturation: 2, 3 and 4 volumes of methanol; heat denaturation: 99°C for 30 min.

collected and digested. For the heat denaturation, an aliquot of human colon homogenate was mixed with equal volume of 50 mM ammonium bicarbonate buffer and then heated at 99°C for 30 min followed by digestion. The remaining immunocapture steps were same and described in the experiment section. The results (Figure 4) show that 4 volume of methanol gives the highest peak area counts, and thus it was selected as the approach of choice. Protein precipitation does not only denature proteins but also largely removes the detergent and HALT protease inhibitors. As a result, the undesired effects of the detergent and HALT protease inhibitors on digestion and immunoaffinity enrichment in the subsequent steps are reduced. Actually the result is due to a combined effect of denaturation, recovery of immunoaffinity enrichment and digestion efficiency, which will be confirmed in the later discussion.

Pellet digestion was optimized with respect to temperature, time and the amount of trypsin required. For direct digestion of biological samples without first removing significant high-abundance proteins, a large amount of trypsin is required to fully digest all of the proteins, since the total amount of proteins are significant. Bovine pancreatic trypsin is commonly used for this type of applications because it is much more affordable at a large quantity. However, it was observed that the peptides, SIL-SSVSDYVNYDIIVR and SIL-LTPAQWFLR completely disappeared when the neat solution of the SIL-peptides was incubated with the native trypsin even for 10 min due to a broadened specificity by autolysis. As a result, sequencing grade modified trypsin was required due to its high specific activity and resistance to autolytic digestion. When the sample size was minimized to 5 mg, 10 µg of sequencing grade modified trypsin was sufficient for sample digestion. For the heat denaturation approach, the amount of trypsin required was also evaluated and the results show that much more trypsin was required to fully digest proteins for the samples involving heat denaturation than the samples involving protein precipitation based denaturation. For heat denaturation, HALT protease inhibitor cocktail and T-PER buffer are not removed, and they have inhibitory effect for digestion. HALT protease inhibitor cocktail was added to lysis buffer prior to homogenization of colon tissue samples, to inhibit protease activity and protect cellular proteins from degradation. Its formulation contains six protease inhibitors AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A. Some of them are actually trypsin inhibitors. As a result, more trypsin is needed to fully digest the colon samples. For pellet digestion, two digestion conditions, 37°C overnight and 60°C for 2 hours, were compared and the results

Figure 5. The effect of digestion conditions on LC-MS/MS peak area count of the surrogate peptide LTPAQWFLR, which is from endogenous S1PR1 in human colon homogenate. Trypsin digestion: 37°C overnight; 60°C for 2 h.

Figure 6. The optimization of the acid elution of the peptides of interest from tosylactivated beads following peptide immunoaffinity enrichment.

are shown in Figure 5. The results show that the overnight digestion at 37°C generated higher digestion yield than the digestion at 60°C for 2 hours.

Since there are multiple cysteines in S1PR1, reduction and alkylation of disulfide bonds prior to trypsin digestion might make the proteolytic cleavage sites more accessible to trypsin. Digestions with and without reduction/alkylation were evaluated for equal volume aliquots of human colon homogenate samples. Reduction was performed by adding 10 µl of 100 mM dithiothreitol in water to the denatured samples followed by incubation at 60°C for 15 min. Alkylation was conducted by adding 10 µl of 250 mM iodoacetamide in water followed by incubation at RT for 20 min in dark. The following digestion and immunocapture procedures were described in the ‘Experimental’ section. The results show that there is no difference in yield of the two surrogate peptides. As a result, reduction and alkylation were not included in the final method.

Prior to immunocapture, the samples were heated at 99°C for 30 min to deactivate trypsin, since trypsin has inhibitory effect on immunocapture. For beads based immunoaffinity enrichment, both protein G beads and tosylactivated beads were evaluated and compared. Tosylactivated beads offered cleaner MRM background noise than protein G beads and was thus used in the final method. A volume of 25 µl of tosylactivated bead suspension, which could be immobilized with 0.125–1.25 µg antibody, provides sufficient binding capacity for immunoaffinity enrichment of the peptides of interest in 100 µl of the samples. The acid elution of the peptides of interest from tosylactivated beads was optimized. Both SIL-LTPAQWFLR and SIL-SSVSDYVNYDIIVR were spiked into PBS buffer. Following peptide immunoaffinity enrichment, acetic acid (1 and 5%) with 0.05% zwittergent were used to elute the peptides of interest. The peak area counts were compared and results are shown in Figure 6. The results show the peak area counts of the peptides with 5% acetic acid elution are 2–3.5 times higher than the peak area counts with 1% acetic acid elution. For immunoaffinity enrichment, we also evaluated and compared the effects of detergents on immunocapture recovery by using SIL peptides. Both SIL-LTPAQWFLR and SIL-SSVSDYVNYDIIVR were spiked into three matrices, PBS buffer, T-PER based cynomolgus monkey colon

Table 3. The effects of detergents on immunocapture recovery for SIL-LTPAQWFLR and SIL-SSVSDYVNYDIIVR (1 nM) in duplicates.

Sample matrix	Peak area count of SIL-LTPAQWFLR	Peak area count of SIL-SSVSDYVNYDIIVR
PBS buffer.1	137,651	25,377
PBS buffer.2	145,841	25,477
Cyno colon homogenate with T-per.1	106,934	3024
Cyno colon homogenate with T-per.2	116,838	3626
Cyno colon homogenate with RIPA.1	18,625	311
Cyno colon homogenate with RIPA.2	10,561	336

Figure 7. Alignment of S1PR1 receptor protein sequences for human, mouse, rat, pig, cynomolgus monkey and dog.

homogenate and RIPA based cynomolgus monkey colon homogenate. Samples were processed by immunoaffinity enrichment as described in the ‘Experimental’ section. The peak area counts were compared and results are shown in Table 3. T-PER is mild detergent while RIPA is a strong detergent. For SIL-LTPAQWFLR, immunocapture recovery is good for T-PER based colon homogenate while significant loss was observed for RIPA based colon homogenate. For SIL-SSVSDYVNYDIIVR, significant loss was observed for both T-PER and RIPA-based colon homogenate. The results suggest that the removal of detergents by using protein precipitation based denaturation is necessary for the subsequent immunocapture.

Assay performance

Due to the endogenous level of biomarkers in authentic matrices, surrogate matrices such as buffer or a corresponding matrix from another species, which do not contain the analytes of interest are commonly used for calibration standards. Since the surrogate peptide, LTPAQWFLR is a common peptide across multiple species including mouse, rat, pig, cynomolgus monkey and dog as shown in Figure 7, colon tissues from these species can’t be used as matrix for calibration standards. As a result, the lysis buffer with 1% HALT protease inhibitor cocktail and 0.1% BSA was chosen as surrogate matrix for calibration standards. Two sets of calibration standards with the recombinant S1PR1 protein and SIL peptide were assessed to address any potential discrepancies. For the first set of calibration standards, human S1PR1 full-length recombinant protein without tag, was spiked and processed following the same procedure as colon samples. For the second set, the SIL peptides were spiked and prepared by peptide immunoaffinity enrichment procedure. These two sets of duplicate calibration standards, which were placed at the beginning and end of the analytical run, were analyzed along with 30 human colon samples. Figure 8 shows the calibration curves of human S1PR1 full-length recombinant protein and SIL peptide. It demonstrates that two curves are well matched, indicating that protein digestion is complete. As a result, the percentage difference of the measured S1PR1 concentration levels between protein curve and peptide curve is within 5.8% for all 30 colon samples. The recombinant proteins are commercially available only for human S1PR1 but not for other species such as mouse, rat, pig, cynomolgus monkey and dog. Therefore, peptide calibration curves need to be used to quantify S1PR1 in other species. Furthermore given that the difficult and high cost associated with generation of multi-transmembrane recombinant proteins, using a peptide calibration standards leads to significant time and cost savings.

The assay performance was further evaluated by immunocapture recovery, matrix effect, precision and accuracy. The average recovery of 91% was determined by comparing the peak area of SIL-LTPAQWFLR between a sample which was spiked prior to immunocapture, and a sample, which was spiked after immunocapture. The matrix effect

Figure 8. Calibration curves of human S1PR1 full-length recombinant protein and SIL-LTPAQWFLR. Calibration curve range is from 7.8 to 500 pM.

Table 4. Accuracy and precision of quality controls for S1PR1 protein and SIL-LTPAQWFLR peptide.

Statistic	Concentration (pM)					
	15.63	31.25	62.5	125	250	500
Quality controls of S1PR1 in 3 replicates						
% DEV	3%	4%	-9%	-3%	7%	2%
% CV	7%	9%	5%	5%	3%	5%
Quality controls of SIL-LTPAQWFLR in 3 replicates						
% DEV	-2%	-6%	2%	-1%	5%	6%
% CV	15%	9%	5%	5%	5%	4%

% CV: The coefficient of variation; % DEV: The percentage deviation; QC: Quality control.

was determined by comparing the peak area of SIL-LTPAQWFLR between a postspiked sample, which was spiked after digestion/immunocapture of colon sample and a neat SIL-LTPAQWFLR sample. The average matrix effect is 87%. The LLOQ of the assay is 7.8 pM and the blank is clean without interference peaks. The calibration curve range is from 7.8 to 500 pM. A linear least-squares regression with weighting factor of $(1/x^2)$ was used [33], and the regression coefficient of the calibration curve was 0.993 for the protein curve and 0.996 for the peptide curve. The accuracy and precision of QCs for both S1PR1 protein and SIL peptide are shown in Table 4. The accuracy and precision of QCs for the protein are within ± 9 and 9%, respectively, and the accuracy and precision of QCs for SIL peptide are within ± 6 and 15%, respectively. The within-run variability for unknown samples were assessed for 3 unknown human colon tissue samples. Each human colon tissue sample (~ 200 mg) was pulverized, and three aliquots of each pulverized sample were processed and analyzed by LC-MS/MS. The within-run variability of the measured concentration levels for three aliquots are 1, 3 and 16%, for three unknown samples respectively.

LC-MS/MS extracted ion chromatograms of human S1PR1 in ~ 5 mg human colon samples are shown in Figure 9. The surrogate peptide LTPAQWFLR with the $566.3 > 917.5$ MRM transition was used for quantitation, and the other peptide and MRM transitions were used for confirmation and specificity. Basal S1PR1 levels for 10 normal human colon samples (~ 5 mg) were measured and the data are shown in Figure 10. Total protein concentration levels were determined for all colon tissue homogenate and the concentration levels of S1PR1 were normalized by the corresponding total protein concentration. This is an essential step for actual biopsy studies as the expected samples will be too small in size to accurately weight out and without a proper normalization the data cannot be properly interpreted. Normalization of tissue protein levels to total protein is a well-established approach.

High sequence homology of S1PR1 was observed between human and other species including cynomolgus monkey, rat, mouse, dog and pig as shown in Figure 7. LTPAQWFLR is a common surrogate peptide across all these species. Therefore, the method for human S1PR1 could be applied for quantitative determination of S1PR1 in other 5 species. It has been successfully applied for analysis of cynomolgus monkey colon tissue samples. It could potentially allow for direct comparison of S1PR1 expression levels among different species.

Since the colon tissue samples were extracted and solubilized by detergents without first isolating cell surface S1PR1 from membrane, this assay is considered for measurement of total S1PR1 levels, which is total of receptors in the membrane and receptors that have been internalized. Isolation of cell surface S1PR1 prior to detergent-based extraction is required in order to measure cell surface S1PR1 by peptide immunoaffinity LC-MS/MS.

Figure 9. LC–MS/MS extracted ion chromatograms of human S1PR1 in ~5 mg human colon samples. The surrogate peptide LTPAQWFLR with the 566.3 > 917.5 MRM transition was used for quantitation. The other peptide and MRM transitions monitored for specificity.

Figure 10. Basal S1PR1 levels for 10 normal human colon samples (~5 mg).

Conclusion

This is the first report of quantitative determination of the GPCR protein, S1PR1 by using peptide immunoaffinity LC–MS/MS. It covers bioanalytical considerations and strategies for GPCR protein quantitation by using LC–MS/MS based methods, including extraction, immunoaffinity enrichment, low sequence coverage and choice of calibrants. Several detergents have been evaluated for extraction of S1PR1 from colon tissue samples. Peptide immunoaffinity enrichment has been employed to specifically isolate the surrogate peptides with high recovery and low matrix effect. It is also demonstrated that with the optimized conditions, protein and peptide calibration curves are well matched and provide the same results for S1PR1 quantitation. A sensitive LC–MS/MS assay with an LLOQ of 7.8 pM has been achieved and the basal S1PR1 levels in small biopsy-sized human colon tissue samples have been determined. Since all the GPCR proteins share the similar structures and challenges, the same strategy and approach have been successfully applied to several other GPCR protein quantitation in our laboratory.

Future perspective

GPCRs represents one of the most important therapeutic target class because of their key roles in transmitting chemical signals into a wide array of different cells and their clinical relevance for a wide range of diseases. However, due to the intrinsic nature of GPCR proteins, the characterizations and quantitative measurements of GPCR proteins are challenging and therefore a lot less is known and published as compared with other proteins. Their intrinsic nature makes it particularly challenging to generate specific antibody reagents for protein immunoaffinity enrichment. In this work, we employed a peptide immunoaffinity LC–MS/MS approach for specifically enriching and detecting surrogate peptides. Our work demonstrates that with optimization, peptide immunoaffinity LC–MS/MS methods can achieve the desired sensitivity for the low abundance GPCR protein, S1PR1. The same strategy could be applied to other GPCR proteins.

A number of bioanalytical challenges and considerations have been addressed, and the assay has been fully optimized to ensure that the assay can reliably measure what intend to be measured and generate meaningful data for the discovery studies. As the assay qualification strategy for early exploratory biomarkers, full validation is not required. However several critical checks have been assessed as part of fit-for-purpose qualification to ensure that the bioanalytical method rigor is suitable for the intended use of the biomarker data in drug discovery. To further improve assay quality and address the issues for matrix difference between samples and calibration standards, in-sample calibration curve using multiple isotopologue reaction monitoring (MIRM) of a stable isotopically labeled analyte will be evaluated and applied for future studies [34,35]. It eliminates the issue of lacking of analyte-free authentic matrices for biomarker quantitation, while keeps samples and calibration standards in the exact same matrices.

The surrogate peptide, LTPAQWFLR is a common peptide in human, cynomolgus monkey, rat, mouse, dog and pig. As a result, the anti-peptide antibodies and LC–MS/MS method for human S1PR1 can be applied for multiple species. Preclinical studies rely heavily on animal models of human disease to assess efficacy and safety risk, and, therefore, biomarker assays are often required for multiple species. Peptide immunoaffinity LC–MS/MS could be one-size-fit-all approach for multiple species in many cases. Another advantage of peptide immunoaffinity enrichment is that it can be applied to formalin-fixed paraffin-embedded (FFPE) tissue samples in pre-clinical or clinical studies where anti-protein based immunoaffinity approach is unlikely to work. It could also be a potential method for measuring total ligand following dosing with the anti-ligand antibody drugs. Peptide immunoaffinity enrichment bypasses issues around generation of a non-inhibitory anti-ligand antibody as capture reagent, which

Executive summary

Background

- The G protein-coupled receptors (GPCRs) are important drug targets and account for more than a third of approved drugs covering wide range of disease types, while numerous novel GPCRs are being studied for the unmet medical needs. Quantitative determination of GPCR expression is thus important for drug discovery and development.
- Due to their hydrophobic nature and intrinsic difficulties of extraction and solubilization, along with low expression levels, quantitative determination of GPCR proteins remains challenging.
- Since all the GPCR proteins share the similar structures, it is important to understand challenges and establish bioanalytical strategies for assay development.
- Due to extremely low abundance and small biopsy-sized colon tissue, a highly sensitive method was required to quantify S1PR1.

Results

- Developed bioanalytical strategies for absolute quantitation of the GPCR protein, S1PR1 in colon tissue samples.
- Developed a highly sensitive peptide immunoaffinity LC–MS/MS method for S1PR1 with LLOQ of 7.81 pM.
- The assay was employed for quantitative determination of endogenous basal levels of S1PR1 in ~ 5 mg human colon tissue samples.

Conclusion

- This is the first report of quantitative determination of S1PR1 in biological matrix by using peptide immunoaffinity LC–MS/MS.
- The assay could be used to quantify S1PR1 in multiple species, since a common peptide is used.
- It is demonstrated that with optimization, peptide immunoaffinity LC–MS methods could achieve the desired sensitivity for the low abundance GPCR protein, S1PR1.
- The same strategy could be applied to other GPCR protein quantitation.

should not compete with drug (antibody) and detection antibody [17]. This could significantly save on time and cost involved in screening for antibody reagents. Peptide immunoaffinity LC–MS/MS is a complementary strategy to anti-protein immunoaffinity assays and is being increasingly used for protein quantitation.

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