ORIGINAL ARTICLE

Validated LC-MS/MS method of Sphingosine 1-phosphate quantification in human serum for evaluation of response to radiotherapy in lung cancer

Xiaohui Tang[1,2], Haisheng Chen[2], Guanxuan Chen[3], Cunxian Duan[2], Qing Fan[2], Hui Li[2], Yanhong Wang [2], Zhijun Li[1,2], Wenna Shi[2] & Yuguo Liu[2]

1 School of Medicine and Life Sciences, University of Jinan Shandong Academy of Medical Sciences, Jinan, China
2 Department of Pharmacy, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, China
3 Department of ICU, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, China

Abstract

Background: Sphingosine 1-phosphate (S1P), a bioactive lipid, has been shown to mediate cancer processes. Therefore, accurate qualitative and quantitative determination is essential. The current assay method is still cumbersome to be of practical use worldwide and the aim of this study was therefore to develop a fast, accurate, precise and efficient LC-MS/MS method for targeted analyses of S1P in serum samples.

Methods: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an established method used for monitoring and analyzing S1P levels in serum. We determined the level of serum S1P in 256 patients with lung cancer and 36 healthy donors, and used Spearman';s rank correlation analysis to evaluate the difference in serum S1P levels between radiotherapy and nonradiotherapy patients.

Results: Standard curves were linear over ranges of 25–600 ng/mL for S1P with correlation coefficient (r^2) greater than 0.9996. The lower limit of quantifications (LLOQs) was 25 ng/mL. The intra- and interbatch precisions and accuracy was less than 10% for S1P. The recoveries of the method were found to be 80%–98%. Serum S1P levels in healthy donors were different from those in patients ($P < 0.001$). Of 256 lung cancer patients, 124 (48.4%) received radiotherapy and were identified to have concomitant low serum S1P levels (222.13 ± 48.63), whereas 132 (51.6%) who had not received radiotherapy were identified to have high levels (315.16 ± 51.06). The serum S1P levels were therefore associated with radiotherapy (Spearman's Rho = -0.653, $P < 0.001$).

Conclusions: Our results indicated that this new LC-MS/MS method is rapid, sensitive, specific and reliable for the quantification of S1P levels in serum samples. The level of S1P in serum samples of patients with lung cancer who received radiotherapy was significantly lower than that in patients who did not receive radiotherapy.

Key points

An improved method was established to quantify S1P levels in human serum by LC-MS/MS, which enabled the change in serum S1P levels in lung cancer patients to be monitored, in combination with radiotherapy, and their clinical significance to be analyzed.

Introduction

Lung cancer is among the leading cause of death from malignancy, and is a great threat to people's lives and health.[1] It has been previously reported that at the time of diagnosis, 75% of cases had advanced disease, with an overall five-year survival rate of only 1%–5%.[2,3] Even though the latest targeted therapies are widely used, first- and second-line chemotherapy are still not effective in treating patients with advanced or recurrent lung cancer.[2] Therefore, development of more effective targeted drugs for lung cancer, or the discovery of a new correlation between radiotherapy or chemotherapy with other tumor-related active substances, may be a new field of research.[4]

Sphingosine 1-phosphate (S1P) is an effective biologically active signaling molecule that plays an important role in a variety of physiological and pathological processes in tumorigenesis and progression.[5–7] Previous studies have linked aberrant S1P signaling to invasion, metastasis, chemo-and radio-resistance in lung cancer cells.[8] Although the specific mechanism has not yet been clarified, it has been reported that S1P can be used not only as a ligand for G-protein coupled receptors (S1PR1–5) outside cells, but also as a messenger in cells, and has not yet been discovered. Based on these characteristics, S1P is proposed as a potential therapeutic or predictive target in lung cancer. As a result, there is an urgent need for standardized, noninvasive, objective, and accurate markers that are sensitive and specific for risk stratification.

A number of detection methods, including ultraviolet (UV), fluorescence (FL), mass spectrometry (MS), and electrochemical detection, have been employed for the determination of S1P in blood samples. Liquid chromatography-tandem mass pectrometry (LC-MS/MS) is now well accepted technology because of greater sensitivity, quantitative and specificity.[9,10] So far, LC-MS/MS methods have been developed only for the determination of the S1P content in plasma.[11–13] The correlative research in serum has so far not been widely reported. Nevertheless, accurate quantification of S1P levels in serum still poses many difficulties to modern LC-MS/MS technology, mainly due to variable effects of a wide variety of biological matrices, as well as the lack of proper matrices free of analytes or samples with known concentrations of analytes.[12–16]

The aim of this study was to develop a fast, accurate, precise and efficient LC-MS/MS method for targeted analyses of S1P in serum samples. The method was used to identify and quantify the levels of serum S1P in patients with lung cancer, and the correlation between serum S1P level and radiotherapy in patients with lung cancer was studied by statistical analysis. This monitoring study may also provide effective data support for radiotherapy sensitivity in patients with lung cancer.

Methods

Reagents and chemicals

Sphingosine 1-phosphate (S1P), and C17-sphingosine 1-phosphate (a 17 carbon analog of S1P, C17-S1P), as shown in Figure 1, were purchased from Avanti Polar Lipids Avanti Polar Lipids (Alabastar, AL, USA). DMSO was obtained from Sigma-Aldrich Trading Co. Ltd. (Shanghai, China). HPLC grade methanol and formic acid were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, USA). All other organic solvents and chemicals were of analytical grades. Deionized water was obtained from a Millipore water purifier. Human serum samples were provided by the Laboratory of Shandong Cancer Research Institute.

LC–MS/MS instrument and conditions

S1P was quantified by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) on Shimadzu LC- 20A (Shimadzu Corp, Japan) and Applied Biosystems API 4000 MS/MS instruments (Applied Biosystems, Foster City, CA). C18 chromatographic column (Agilent Eclipse Plus C18, 2.1 mm × 50 mm, 5 μm) was used for chromatographic separation. Data acquisition and process were by Metabo 1.6.3 controlling software. The mobile phase was composed of water 0.1% formic acid (mobile phase A) and methanol 0.1% formic acid (mobile phase B). The flow rate was set at 500 μL/min. The running time of each injection was 3.7 minutes, which was better than than that reported in a previous study.[17] A mass spectrometer was operated in the positive ion mode (ESI+) with an electrospray voltage of 5500 V at 400°C of the heated capillary temperature. Nitrogen was used as the air curtain gas (20 psi), atomizing gas (30 psi), auxiliary gas (60 psi) and collision gas (4 psi). The multiple reaction monitoring (MRM) was set up for quantitative. The precursors were optimized for product ions and collision energies as: m/z $366.4 \rightarrow 250.2$ for C17-S1P and m/z $380.4 \rightarrow 264.4$ for S1P. Typical production mass spectra of the $[M + H]^+$ ion of S1P and C17-S1P
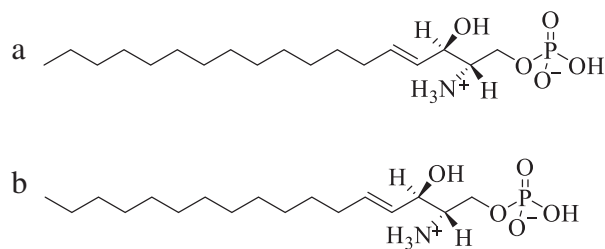
Figure 1 Chemical structures of (**a**) S1P; and (**b**) C17-S1P.
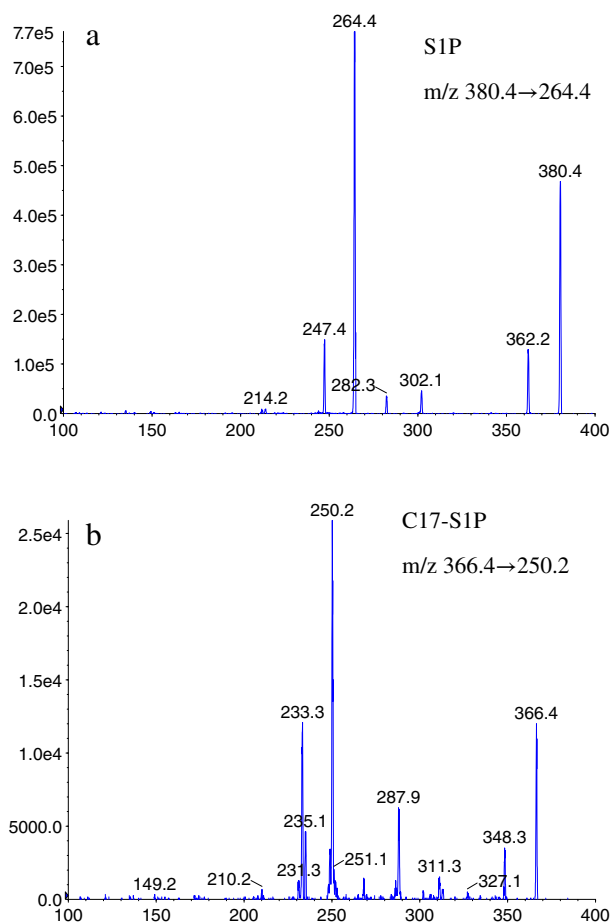
Figure 2 Representative full-scan product ion mass spectra of [M + H] $^+$ ions of (**a**) S1P; and (**b**) C17-S1P.

are shown in Figure 2. Dwell time was 100 ms, DP was 74 v, EP was 10 v, CE was 22 v for S1P and 23 v for C17-S1P, CXP was 15 v.

Stock solution, calibration standards, lower limit of quantification (LLOQ) and quality control (QC) preparation

S1P and C17-S1P solutions at a concentration of 1 mg/mL were prepared in dimethyl sulfoxide (DMSO)/concentrated hydrochloric acid (HCl) (100:2, v/v). All stock solutions were stored at −20°C. Methanol and water (1:1, v/v) were then added to the stock solution to make a standard working solution. Calibration standards were prepared by adding corresponding working solutions in surrogate matrix to obtain final concentrations of standard samples from 25–600 ng/mL for S1P. The lower limit of quantification (LLOQ) and quality control (QC) samples were individually and similarly prepared in surrogate matrix. The concentrations of LLOQ samples and three levels of QCs samples were 25, 75, 200, 480 ng/mL, respectively.

Sample collection and analysis

All serum samples were obtained from Shandong Cancer Hospital and Institute. All samples and solutions were brought to room temperature before quantification. A protein precipitation method was used to extract the analytes from serum samples. We chose 4% BSA as a surrogate matrix. We then mixed 20 μL serum samples with 200 μL methanol solution containing 25 ng/mL of internal standard at room temperature to obtain final samples, vortex-mixed for one minute, and then centrifuged at 12 000 revolutions per minute for 10 minutes. An aliquot of 1 μL of the supernatant was injected into the LC–MS/MS system for analysis.

Methodological consistency verification

A total of 20 randomly collected plasma and serum samples from lung cancer patients or healthy donors were tested for methodological consistency verification. Plasma and serum samples were collected from the same patient at

the same time. Plasma samples were tested according to the methods detailed in a previous study.[9]

Method validation

Selectivity

Selectivity was used to distinguish the endogenous components of the target analyte and the internal standard from the matrix or other components in the sample. We used six batches of blank surrogate substrates to demonstrate selectivity, which were analyzed and evaluated for interference. When the response of the interference component in blank surrogate matrix was measured to be less than 20% of the analyte response in LLOQ, and less than 5% of the internal standard response, the selectivity was determined to be good.

Accuracy and precision

According to the Food and Drug Administration (FDA) guidelines,[18] the precision and accuracy were determined by assessing the QC samples of four concentration levels of S1P, respectively. Each level of concentration contained six samples. These samples were repeatedly analyzed six times on one day, and then on three consecutive days (intra- and interday). Precision was described as relative standard derivative (RSD) and accuracy was described as relative deviation.

Recovery

Recovery was assessed by comparing mean peak areas of samples spiked before and after extraction, and two concentrations of medium and high were selected from the QC concentration level for evaluation, and each sample was repeated in one day.

Matrix effect

Matrix was assessed by using six batches of surrogate matrix, and the matrix factors of SIP and C17-S1P were calculated by calculating the ratio of peak area in the presence of blank surrogate matrix (extracted from blank surrogate matrix and added analyte and internal standard) to the corresponding peak area of without surrogate matrix (analyte and pure solution of internal standard). Furthermore, the matrix factor normalized by the internal standard was calculated by dividing the matrix factor of the analyte by the matrix factor of the internal standard. The coefficient of variation of the normalized matrix factor calculated from six batches of surrogate should be within 15%. The determination was carried out at low and high concentrations, respectively.

Standard curves

The standard curve verification was to add the analyte (and internal standard) of known concentration to the blank surrogate matrix to prepare the calibration standard sample of each concentration. The range of the standard curve covered the expected concentration range, which was determined by the quantitative lower limit and the quantitative upper limit. Three standard curves were repeatedly evaluated and their linear coefficients were analyzed.

Stability

The stability was analyzed after pretreatment and 12 hours in the injection chamber using low and high concentration quality control samples. The deviations of the mean predicted concentrations of the low and high concentration quality control samples from the nominal concentrations were used as an indicator of the stability of the analyte. To establish the stability of the analyte, the deviations of the mean measured concentrations of the samples should be within $\pm15\%$ of the nominal concentrations.

Patient selection and serum sample collection

In this study, serum samples were collected from 36 healthy donors and 256 patients diagnosed with lung cancer attending the Shandong Cancer Hospital between January 2018 and November 2019. Serum samples were taken on the day of peripheral blood collection. Hemolytic specimens were excluded. All participants provided their written informed consent.

The inclusion criteria were: patients must be aged between 20 to 75 years and diagnosed with lung cancer as defined by the National Comprehensive Cancer Network (NCCN) guidelines.[19] Patients should not have received any other treatment for six months, and received radical radiotherapy in once daily fractions of 2 Gy over a six week period. Serum samples were collected within one week after treatment. Tumor staging was estimated according to the AJCC Cancer Staging Handbook of the American Joint Committee on Cancer, 2010.[20]

The exclusion criteria were: The patients suffer from any other diseases, including immune-deficiency, endocrine, metabolic and blood disorders.

S1P quantification

The validated method was used for the quantification of S1P in the serum of 256 lung cancer patients and 36 healthy donors. All samples were obtained from Shandong Cancer Hospital and Institute. Samples treatment and quantification methods were in accordance with validated method.

Statistical analysis

All statistical tests were performed using SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA). Continuous variables were expressed as means and standard deviation (SD) or medians and the interquartile range. The Kolmogorov-Smirnov test was used to examine the distribution of the data. The data between two groups were compared using the independent samples *t*-test. The degree of association between variables was measured by the Spearman's Rho test. A *P*-value ≤0.05 was considered statistically significant. The figures were created using GraphPad Prism 7 software.

Results

Methodological consistency verification

A total of 20 randomly collected plasma and serum samples were tested for methodological consistency verification. The results are shown in Figure 3. The results indicated that the differences in S1P content in serum and plasma were consistent for the same sample, which proved that this was a credible method.

Validation

Selectivity

Six different batches of blank surrogate matrix samples were included to assess the selectivity, which were analyzed and evaluated for interference. The results of the selectivity test are shown in Figure 4. The response of the interference component in blank surrogate matrix was less than 20% of the response of the analyte in LLOQ and less than 5% of the internal standard response.
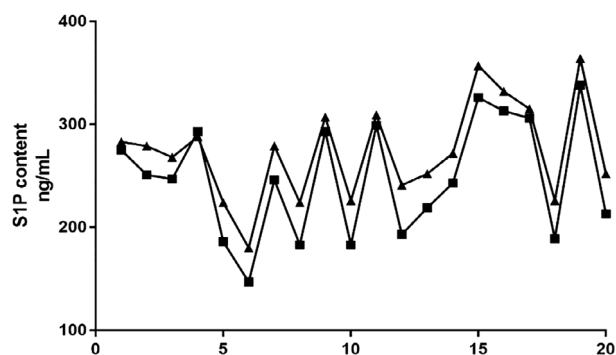
Figure 3 The content of S1P levels in 20 randomly collected plasma and serum samples. ─▲─ serum and ─■─ plasma

Accuracy and precision

The accuracy and precision of the method were evaluated by analyzing four sets of QC samples. Each level of concentration contained six samples. The results are shown in Table 1. The intra- and interbatch precisions and accuracy were less than 10%.

Recovery

Recovery was assessed by comparing mean peak areas of samples spiked before and after extraction, two concentrations of medium and high were selected from the QC concentration level for evaluation, and each sample was repeated. The results are shown in the Table 2. The extraction recovery rate of S1P was 80%–98%, C17-S1P was 88%–95%, and the RSD was controlled within 6%.

Matrix effect

For the matrix effect test, the peak area of a certain concentration of S1P added to the replacement matrix and the peak area of the pure solution containing the same concentration of S1P were compared. C17-S1P also conducted the matrix effect test. The results are shown in Table 3. The RSD was controlled within 6%, and it indicated that the methodology can be used to determine the sample content without matrix effects.

Standard curves

The standard curve of the replacement matrix was measured at seven concentration points. As shown in Figure 5, the standard curve showed good linearity, and the calibration curve concentration ranged from 25 to 600 ng/mL. The relative standard deviation value was controlled within 5.1%. The representative regression equation was $y = 0.00377 x + 0.00378$ and the correlation coefficient $r = 0.9996$.

Stability

We used low and high QC samples to assess stability. The stability of the injection chamber 12 hours was investigated. We compared the results with the stability results of freshly made samples. The stability results are shown in Table 4. The results indicated that there was good stability within 12 hours of the injection chamber, and the RSD was within 4% and the average deviation less than 15%.

Validated method of monitoring serum S1P concentration in biological samples

The validated method was successfully used for the quantification of S1P in the serum of 256 lung cancer patients and 36 healthy donors. Serum S1P levels in healthy donors (123.64 ± 24.11) were different from those in patients (270.46 ± 68.19, $P < 0.001$). Of 256 lung cancer patients,
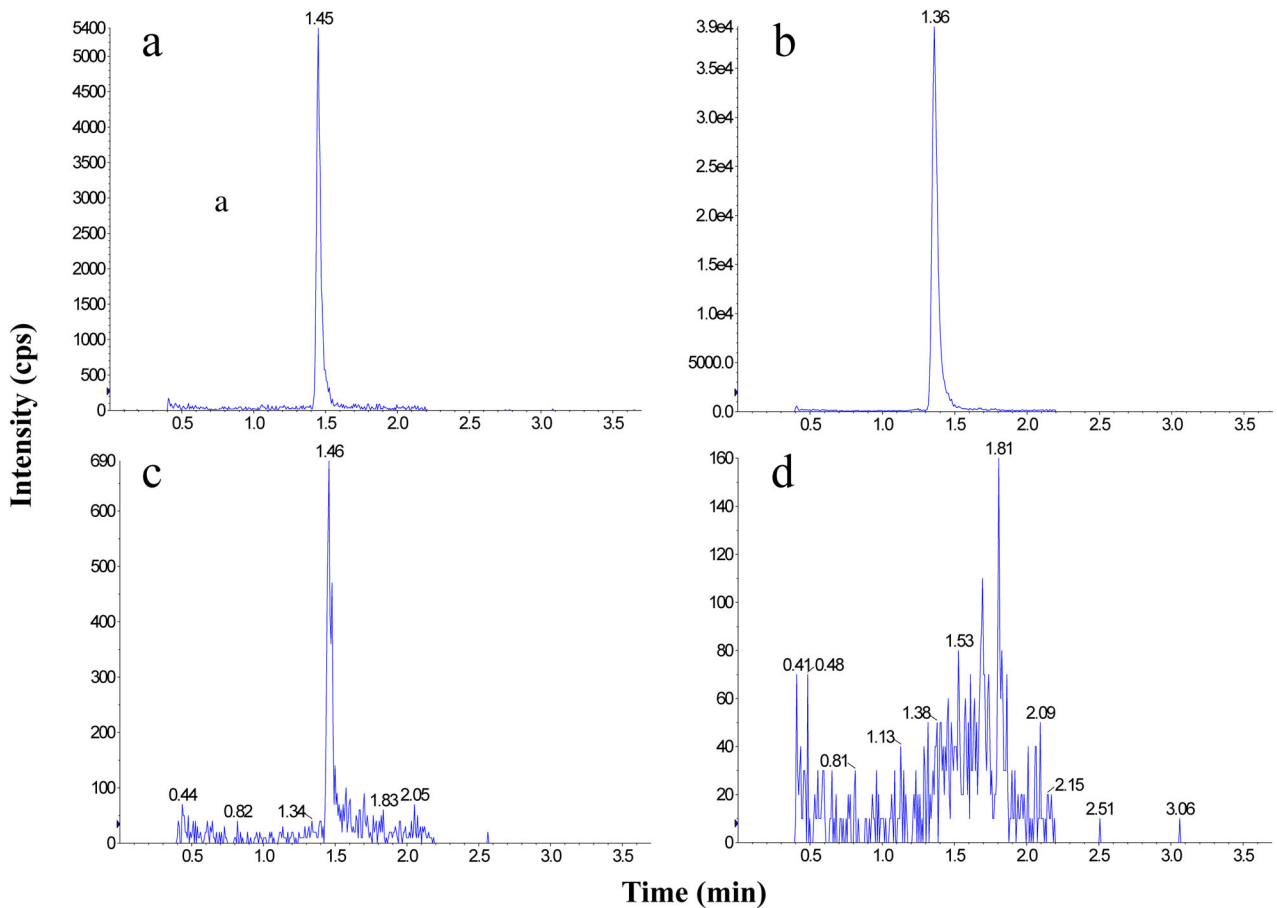
Figure 4 MRM chromatograms of (**a**) LLOQ of S1P;, (**b**) LLOQ of C17-S1P; (**c**) blank surrogate matrix of S1P; and (**d**) blank surrogate matrix of C17-S1P.

124 (48.4%) received radiotherapy and were identified to have concomitant low serum S1P levels (222.13 ± 48.63), whereas 132 (51.6%) who had not received radiotherapy were identified to have high levels (315.16 ± 51.06, $P < 0.001$). There was a significant difference in S1P content between different age groups (Spearman's Rho = 0.188, $P = 0.003$) and different degree of differentiation groups (Spearman's Rho = 0.288, $P = 0.003$). There were no statistical differences in serum S1P levels when comparing the results of gender, smoking status and tumor size. The results of the independent samples *t*-test are shown in Figure 6.

Table 1 Precision (CV, %) and accuracy data of S1P ($n = 6$)

| Compound | Nominal concentration (ng/mL) | Precision | | Accuracy (%) |
		Mean ± SD	RSD (%)	
Intrabatch				
S1P	25	23.9 ± 0.756	3.16	−4.45
	75	77.2 ± 2.42	3.13	2.8
	200	209.2 ± 3.31	1.58	4.5
	480	492 ± 7.5	1.52	2.5
Interbatch				
S1P	25	26.2 ± 2.05	7.81	5
	75	76.5 ± 2.3	3.01	2
	200	205.2 ± 7.88	3.84	2.5
	480	497 ± 11.71	2.36	3.5

RSD, relative standard deviation; S1P, D-erythro-sphingosine 1-phosphate; SD, standard deviation.

Table 2 The extraction recovery for S1P and C17-S1P (*n* = 6)

Compound	Nominal concentration (ng/mL)	Recovery (mean ± SD, %)	RSD (%)
S1P	75	92.9 ± 5.49	5.9
	480	94.7 ± 2.14	2.26
C17-S1P	75	90.7 ± 1.24	1.4
	480	91.9 ± 2.08	2.26

C17-S1P, C17-d-erythro-sphingosine 1-phosphate.

Table 3 Matrix effect for S1P and C17-S1P (*n* = 6)

| Compound	Nominal concentration (ng/mL)	Matrix effect (mean ± SD,%)	RSD (%)
S1P	75	103.71 ± 3.67	3.54
	480	101.28 ± 4.60	4.54
C17-S1P	75	96.63 ± 0.93	0.96
	480	101.76 ± 4.30	4.23

Level of serum S1P in lung cancer patients treated with radiotherapy

A total of 124 (48.4%) lung cancer patients received radiotherapy, and their S1P levels were 222.13 ± 48.63. A total of 132 (51.6%) lung cancer patients did not receive radiotherapy, and their S1P levels were 315.16 ± 51.06. The level of serum S1P is closely related to whether the patients receive radiotherapy or not. (Spearman's Rho = −0.653,

$P < 0.001$). The demographic and clinical characteristics are summarized in Table 5.

Discussion

S1P is a biologically active lipid mediator that is abundant in blood.[12] Several methods have been previously studied for the quantification of low-abundant S1P in biological samples.[11,12,21] The enzymatic method was based on the use of radiolabeled substrates [γ-32P] ATP, followed by thin layer chromatography (TLC) separation, and the radioactive spots were visualized by autoradiography.[22] The high-performance liquid chromatography (HPLC) was used for quantitative analysis of S1P. S1P was dephosphorylated with alkaline phosphatase prior to fluorescent derivatization, and the fluorescent derivatives were then separated using HPLC with detection by a spectrofluorometer.[9] There has been no evidence to prove the difference between serum and plasma in previous experiments. In this study, we verified the plasma and serum of 20 samples and found that the changes of S1P content in serum and plasma were consistent. On the basis of our analysis and previous work, we have established a method of extracting analytes from serum samples by protein precipitation with C17-S1P as the internal standard, which is simpler and more sensitive than HPLC. The mobile phase regulated chromatography

Figure 5 Standard curves for quantification of S1P with C17-S1P as the internal standard, respectively.

$$y=0.0037x+0.00378 \ (r=0.9996)$$
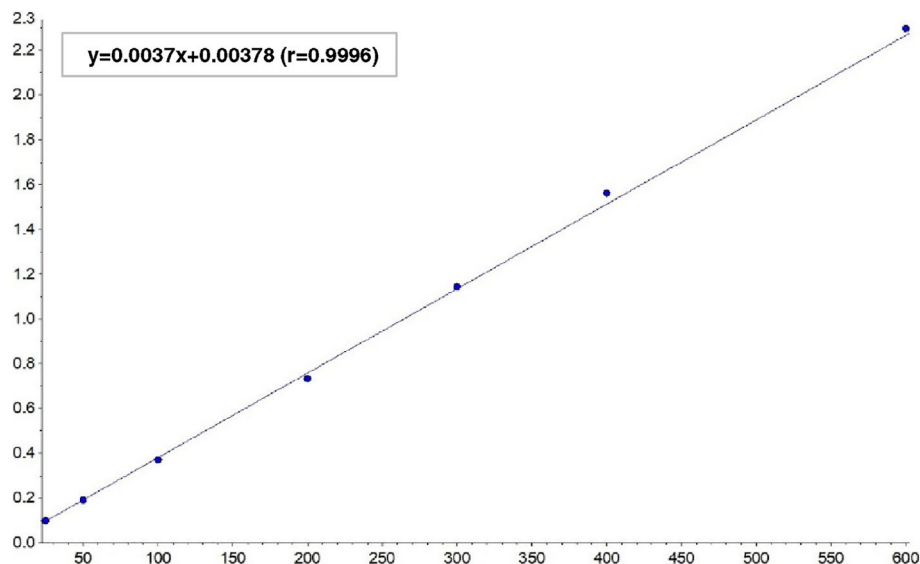
Table 4 Stability of the injection chamber 12 hours

Stability condition	Nominal concentration (ng/mL)	Mean ± SD	RSD (%)	Deviation (%)
Injection chamber stability (injection chamber stay 12 hours)	75	77.9 ± 2.836	3.64	4
	480	522 ± 13.8	2.64	9
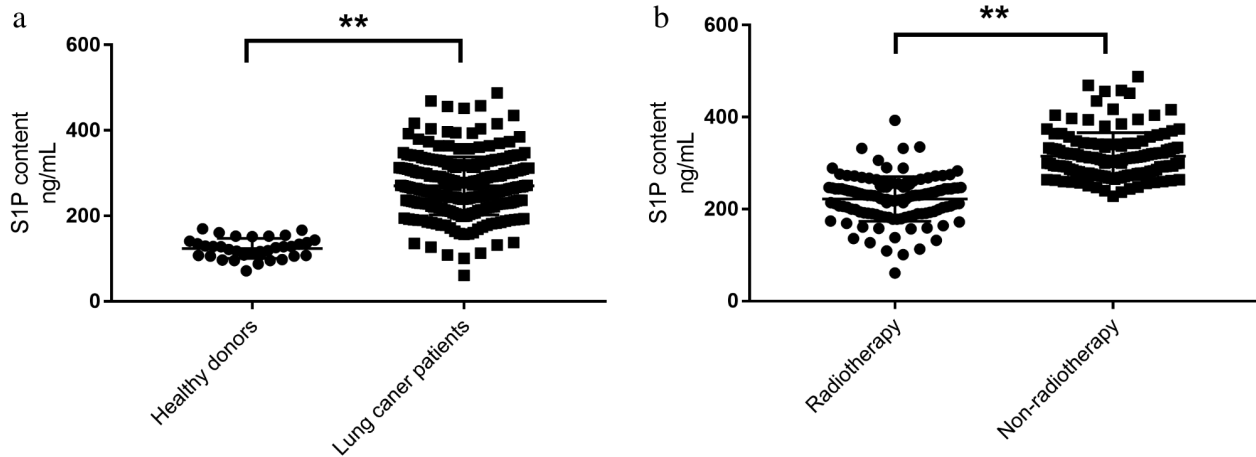
Figure 6 Independent samples *t*-test and Spearman's Rho test. (**a**) Serum S1P levels in healthy donors (*n* = 36) and lung cancer patients (*n* = 256). (**b**) Serum S1P levels in lung cancer patients with radiotherapy (*n* = 124) and nonradiotherapy (*n* = 132). *P*-values were calculated with *$^*P$ < 0.05; $^{**}P$ < 0.001.

Table 5 Baseline characteristics of patients i

Characteristics		Number of cases (*n* = 256)	Median with interquartile range	*P*-value
Age (years)	<61	108 (42.2%)	276.000 (273.072–298.876)	0.003
	> = 60	148 (57.8%)	260.500 (246.926–268.303)	
Gender	Male	144 (56.3%)	268.500 (259.341–279.627)	0.714
	Female	112 (43.7%)	269.500 (257.820–288.124)	
Smoking status	Yes	109 (42.6%)	262.000 (253.981–290.223)	0.852
	No	147 (57.4%)	271.000 (260.556–279.599)	
Tumor size (mm)	<50	138 (53.9%)	264.000 (259.129–282.450)	0.934
	> = 50	118 (46.1%)	272.500 (257.834–282.335)	
Degree of differentiation	Low	25 (9.8%)	231.600 (207.600–255.600)	0.003
	High	201(90.2%)	274.671 (265.859–283.483)	
Radiotherapy	Yes	124(48.4%)	222.138(213.458–230.819)	0.000
	No	132(51.6%)	315.158 (306.399–323.917)	

behavior and appropriate ionization. Compared with the previous method of using acetonitrile, the mobile phase consisted of methanol and water (0.1% formic acid) in different proportions because formic acid enhanced sensitivity and improved peak shapes. Each run was completed within 3.7 minutes, which is an improvement on previous research. We tried different surrogate matrices and finally selected 4% BSA as the surrogate matrix, which effectively eliminated matrix effects, and ensured the methodology with better selectivity, accuracy and precision. Last but not least, serum is more convenient and simpler to collect, handle, store, etc, than plasma in a clinical environment According to the FDA guidelines, we verified selectivity, accuracy and precision, recovery, matrix effect, the calibration curve and stability of this methodology.

S1P is considered to regulate many physiological processes, and act as a signaling molecule in cells. There is substantial evidence for a role for S1P in promoting transformation, epithelial mesenchymal transition and invasiveness, cancer cell survival, replicative immortality, tumour neovascularisation and aerobic glycolysis; the so-called hallmarks of cancer.[23,24] Thus, there is an urgent need to establish an efficient and reliable assay to quantify the S1P. At present, there is a lack of data on the relationship between the influencing factors of tumor treatment and monitoring results of S1P concentration.[25–28] Previous studies have focused on the establishment of a certain method and did not apply it to the treatment and differences in specific cancer patients. We developed the LC-MS/MS method for sensitive mass spectrometric quantification of S1P in the biological samples of lung cancer patients. The verification method we established was used for the quantification of serum S1P levels in 256 lung cancer patients. We analyzed the results of these patients with lung cancer and found that there was a negative correlation and significant difference in serum S1P levels between

radiotherapy and nonradiotherapy patients (Spearman's Rho = −0.653, $P < 0.001$). The results showed that the S1P levels were also related to degree of differentiation in lung cancer patients (Spearman's Rho = 0.288, $P = 0.003$). The serum S1P concentration of patients with low differentiation was significantly lower than patients with high differentiation. It is suggested that the level of serum S1P in patients with lung cancer may be a predictive marker of malignant tumor differentiation and radiation sensitivity. Interestingly, we found that there was a significant difference in S1P content between different age groups (Spearman's Rho = 0.188, $P = 0.003$). This result is different from previous studies which considered that there was no relationship between S1P content and age groups.[29,30] To arrive at this conclusion, we speculated that it may be related to sample size, or sample collection limitations.

According to some studies, intravenous continuous infusion or peritoneal injection of S1P has been shown to contribute to decrease chemotherapy and radiotherapy induced apoptosis on primordial follicles in animals.[31,32] Our results showed that the levels of serum S1P in patients with lung cancer receiving radiotherapy were lower than that in patients without radiotherapy, which was consistent with the trend reported in the literature. Based on the evidence currently available, S1P might play a vital role in the lung cancer radiotherapy. By studying serum S1P levels correlation to radiotherapy, we speculate that S1P may be a predictor of radiation sensitivity that can be monitored in lung cancer patients. However, the exact mechanism of action has not been fully elucidated, and further research and exploration is needed. With regard to a more specific treatment plan and radiation dose of radiotherapy, it will continue to be carried out in our future research. The challenge for us is to formulate a well-designed experimental program, and long-term patient dynamic tracking with the application of the latest research in order to provide more effective guidance for clinical treatment in the future. Our findings still have to be confirmed in various centers with a larger sample size, and follow-up samples to explore the prediction of prognosis in patients with lung cancer by serum S1P concentration. However, the novelty of the method and the results of this study might offer new insights into the mechanisms of action of radiotherapy, and provide a new strategy for predicting radiosensitivity.

In conclusion, we have developed and validated a simple, specific, and reproducible LC–MS/MS method for the quantification of S1P in human serum that is suitable for clinical sample analysis. This method demonstrated good linearity over the concentration range of 25 to 600 ng/mL. The sample handling and storage conditions of the method had no impact on the stability of S1P. The intra- and interday accuracy and precision of the calibration curves and QC samples complied with FDA acceptance criteria for bioanalytical method validation. We finally selected 4% BSA as the surrogate matrix, which effectively eliminated the matrix effects, and ensured methodology with better selectivity accuracy and precision. Each run was completed within 3.7 minutes, which was an improvement on that reported in previous research. The S1P levels decreased in lung cancer patients' serum samples treated with radiotherapy, and the content of serum S1P was negatively correlated with radiotherapy. It is hoped that this study might provide new ideas for the application of basic research of S1P in clinical treatment and for S1P to be used in the evaluation of response to radiotherapy in lung cancer. Further studies are recommended to validate the findings of this study.

Acknowledgments

Disclosure

No authors report any conflict of interest.

References

1 Shen HS, Wen SH. Effect of early use of Chinese herbal products on mortality rate in patients with lung cancer. *J Ethnopharmacol* 2018; **211**: 1–8.

2 Guan S, Liu YY, Yan T, Zhou J. Inhibition of ceramide glucosylation sensitizes lung cancer cells to ABC294640, a first-in-class small molecule SphK2 inhibitor. *Biochem Biophys Res Commun* 2016; **476** (4): 230–6.

3 Jie Y. ABC294640, a sphingosine kinase 2 inhibitor, enhances the antitumor effects of TRAIL in non-small cell lung cancer. *Cancer Biol Therapy* 2015; **8** (16): 1194–204.

4 Willoughby CE, Jiang Y, Thomas HD *et al.* Selective DNA-PKcs inhibition extends the therapeutic index of localized radiotherapy and chemotherapy. *J Clin Invest* 2019; **130**: 258–71.

5 Soriano JM, González L, Catalá AI. Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B1. *Prog Lipid Res* 2005; **44** (6): 345–56.

6 Chalfant EC. Sphingosine 1-phosphate and ceramide 1-phosphate: Expanding roles in cell signaling. *J Cell Sci* 2005; **118** (20): 4605–12.

7 Huang Y-L, Huang W-P, Lee H. Roles of sphingosine 1-phosphate on tumorigenesis. *World J Biol Chem* 2011; **2** (2): 25–34.

8 Wang Y, Shen Y, Sun X, Hong TL, Huang LS, Zhong M. Prognostic roles of the expression of sphingosine-

1-phosphate metabolism enzymes in non-small cell lung cancer. *Transl Lung Cancer Res* 2019; **8** (5): 674–81.

9 Lan T, Bi H, Liu W, Xie X, Xu S, Huang H. Simultaneous determination of sphingosine and sphingosine 1-phosphate in biological samples by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2011; **879**: 520–6.

10 Gong ZS, Wu ZH, Xu SX *et al*. A high-throughput LC-MS/MS method for the quantification of four immunosuppressants drugs in whole blood. *Clin Chim Acta* 2019; **498**: 21–6.

11 Saigusa D, Okudaira M, Wang J *et al*. Simultaneous Quantification of Sphingolipids in Small Quantities of Liver by LC-MS/MS. *Mass Spectrom* 2014; **3**: S0046.

12 Frej C, Andersson A, Larsson B *et al*. Quantification of sphingosine 1-phosphate by validated LC-MS/MS method revealing strong correlation with apolipoprotein M in plasma but not in serum due to platelet activation during blood coagulation. *Anal Bioanal Chem* 2015; **407** (28): 8533–42.

13 Mirzaian M, Wisse P, Ferraz MJ *et al*. Accurate quantification of sphingosine-1-phosphate in normal and Fabry disease plasma, cells and tissues by LC-MS/MS with (13)C-encoded natural S1P as internal standard. *Clin Chim Acta* 2016; **459**: 36–44.

14 Liu Z, Tu MJ, Zhang C, Jilek JL, Zhang QY, Yu AM. A reliable LC-MS/MS method for the quantification of natural amino acids in mouse plasma: Method validation and application to a study on amino acid dynamics during hepatocellular carcinoma progression. *J Chromatogr B Analyt Technol Biomed Life Sci* 2019; **1124**: 72–81.

15 Egom EE, Fitzgerald R, Canning R, Pharithi RB, Murphy C, Maher V. Determination of Sphingosine-1-Phosphate in Human Plasma Using Liquid Chromatography Coupled with Q-Tof Mass Spectrometry. *Int J Mol Sci* 2017; **18** (8): 1–15.

16 Liu X, Hoene M, Yin P *et al*. Quality control of serum and plasma by quantification of (4E,14Z)-Sphingadienine-C18-1-Phosphate uncovers common preanalytical errors during handling of whole blood. *Clin Chem* 2018; **64** (5): 810–9.

17 Schmidt H, Schmidt R, Geisslinger G. LC-MS/MS-analysis of sphingosine-1-phosphate and related compounds in plasma samples. *Prostaglandins Other Lipid Mediat* 2006; **81** (3): 162–70.

18 Berndt ER. Food and Drug Administration (FDA). *Debra Littlejohn Shinder Thomas W Shinder* 2008; **56** (1): 323–4.

19 Ettinger DS, Wood DE, Aggarwal C *et al*. NCCN Guidelines Insights: Non-Small Cell Lung Cancer. *J Natl Compr Canc Netw* 2019; **17** (12): 1464–72.

20 Edge SB, Compton CC. The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 2010; **17** (6): 1471–4.

21 Mirzaian M, Wisse P, Ferraz MJ *et al*. Accurate quantification of sphingosine-1-phosphate in normal and Fabry disease plasma, cells and tissues by LC-MS/MS with (13)C-encoded natural S1P as internal standard. *Clinica chimica acta. Int J Clin Chem* 2016; **459**: 36–44.

22 Edsall LC, Spiegel S. Enzymatic measurement of Sphingosine 1-Phosphate. *Anal Biochem* 1999; **272** (1): 80–6.

23 Pyne S, Adams DR, Pyne NJ. Sphingosine 1-phosphate and sphingosine kinases in health and disease: Recent advances. *Prog Lipid Res* 2016; **62**: 93–106.

24 Maczis M, Milstien S, Spiegel S. Sphingosine-1-phosphate and estrogen signaling in breast cancer. *Adv Biol Regul* 2016; **60**: 160–5.

25 Lee HM, Lo KW, Weib W *et al*. Oncogenic S1P signalling in EBV-associated nasopharyngeal carcinoma activates AKT and promotes cell migration through S1P receptor 3. *J Pathol* 2017; **242** (1): 62–72.

26 Fettel J, Kühn B, Guillen NA *et al*. Sphingosine-1-phosphate (S1P) induces potent anti-inflammatory effects in vitro and in vivo by S1P receptor 4-mediated suppression of 5-lipoxygenase activity. *FASEB J* 2019; **33** (2): 1711–26.

27 Panneer SS, De Palma RM, Oaks JJ *et al*. Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation. *Sci Signal* 2015; **8** (381): ra58.

28 Bouquerel P, Gstalder C, Müller D *et al*. Essential role for SphK1/S1P signaling to regulate hypoxia-inducible factor 2α expression and activity in cancer. *Oncogenesis* 2016; **5**: e209.

29 Moritz E, Wegner D, Groß S *et al*. Data on subgroup specific baseline characteristics and serum sphingosine-1-phosphate concentrations in the study of health in Pomerania. *Data Brief* 2017; **12** (C): 46–50.

30 Winkler MS, Nierhaus A, Holzmann M *et al*. Decreased serum concentrations of sphingosine-1-phosphate in sepsis. *Crit Care* 2015; **19**: 372.

31 Mumusoglu S, Turan V, Uckan H, Suzer A, Sokmensuer LK, Bozdag G. The impact of a long-acting oral Sphingosine-1-phosphate analogue on ovarian aging in a rat model. *Reprod Sci* 2017; **25** (10): 193371911774137.

32 Venkataraman K, Lee YM, Michaud J *et al*. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. *Circ Res* 2008; **102** (6): 669–76.