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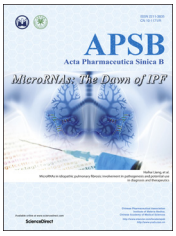

ORIGINAL ARTICLE

Pharmacokinetics of H002, a novel $S1PR_1$ modulator, and its metabolites in rat blood using liquid chromatography–tandem mass spectrometry

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Metabolite;
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Periphery blood lymphocyte

Abstract A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous determination of H002 and its phosphorylated metabolite, H002-P and hydroxylated metabolite H002-M, in rat blood. H001, an analogue of H002, was used as the internal standard. Blood samples were prepared by simple protein precipitation. The analytes and internal standard were separated on a Zorbax SB-C18 column with a gradient mobile phase consisting of methanol and water containing 0.1% formic acid at a flow rate of 0.2 mL/min with an operating temperature of 20 °C. The detection was performed on a triple quadrupole tandem mass spectrometer with positive electrospray ionization in multiple-reaction monitoring mode. Linear detection responses were obtained from 0.2–100 ng/mL for H002 and H002-M, while 0.5–100 ng/mL for H002-P. The intra- and inter-day precision (RSD%) was within 11.76%, with the accuracy (RE%) ranging from –9.84% to 9.12%. The analytes were shown to be stable during sample storage, preparation and analytic procedures. The method was applied to determine the pharmacokinetics of H002 in rats, and a preliminary study showed that the pharmacokinetics of H002 correlated with its biological effect on peripheral blood lymphocytes.

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1. Introduction

Sphingosine-1-phosphate (S1P, Fig. 1) is a bioactive sphingolipid metabolite enriched in blood and lymph, recognized as a critical regulator in multiple physiological pathways, such as cell growth and suppression of apoptosis through interactions with G-protein-coupled receptors[1–3]. As an identified S1P receptor (S1PR), $S1PR_1$ was known to modulate the migration of cells between vascular and lymphatic compartments, demonstrating its potential role as a therapeutic target of autoimmune diseases.

FTY720, an analogue of S1P, could be reversibly phosphorylated to FTY720-P *in vivo* yielding its active form, which could prevent lymphocyte egress from lymphoid tissues and circulation to the periphery[4]. A rapid and detectable reduction of peripheral blood lymphocytes (PBL) count, along with an increasing number of lymphocytes in secondary lymphoid organs (SLO) is confirmed after oral administration of FTY720, presumably indicating its major immunomodulatory mechanism[5,6]. However, severe adverse effects, such as bradycardia and hypertension, may jeopardize a patient's life since FTY720-P binds to $S1PR_{3–5}$ as well as $S1PR_1$, raising the risk in the cardiovascular system[7]. In addition, FTY720 has a quite long elimination half-life in multiple species and requires weeks and even months to allow full recovery of immunological effects, provoking clinical concern as to patients' susceptibility to infections[8–12]. Therefore, optimizing novel $S1PR_1$ agonizts with better selectivity and pharmacokinetic properties seems crucial to achieve satisfactory therapy.

Amino-propylene-glycol derivatives have been recently shown to be promising immune-modulators with lower potency for the $S1PR_3$ receptor, attenuating the risk of adverse effects[13,14]. Among them, H002 (2-amino-2-(2-(4′-(2-ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)ethyl)propane-1,3-diol, previously named SYL927, Fig. 1), a synthetic chemical entity, was found to be reversibly phosphorylated to H002-P (Fig. 1) *in vivo*, and showed receptor selectivity for $S1PR_1$ and less effect on the cardiovascular system as compared to FTY720[15]. The hydroxylated metabolite H002-M (Fig. 1) showed little effect on S1PR. Furthermore, pharmacodynamic studies of H002 revealed a promising therapeutic effect in an experimental autoimmune psoriasis model.

Despite extensive research on the pharmacodynamics of H002, little is known about its pharmacokinetics. The introduction of a phosphate group increased the polarity of H002-P significantly, resulting in decreased solubility in organic solvents compared to the parent drug and its hydroxylated metabolite. Therefore, it is challenging to develop a reliable method to obtain the satisfactory recovery and sensitivity for each analyte. The objective of this study was to develop a simple, sensitive, and accurate LC–MS/MS method for the simultaneous determination of H002, H002-P and H002-M in rats after oral administration of H002, along with measurement of PBL counts to illustrate the relationship between a decrease in PBL counts and drug concentration in blood.

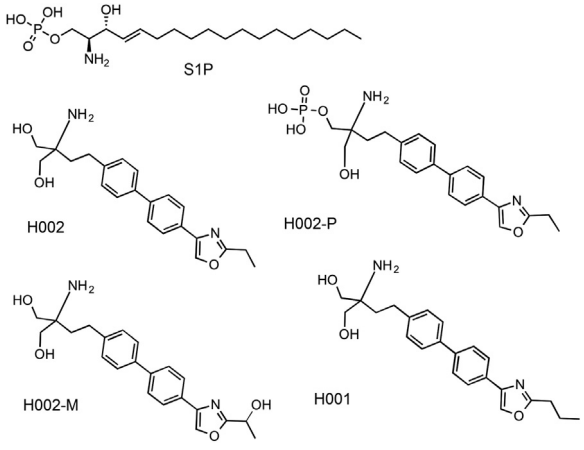

Figure 1 Chemical structures of S1P, H002, H002-P, H002-M and H001 (IS).

2. Experimental

2.1. Chemicals and reagent

H002 (purity 99.1%), H002-P (purity 96.4%), H002-M (purity 90.0%) and the internal standard (IS) H001 (2-amino-2-(2-(4′-(2-propyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)ethyl)propane-1,3-diol, Fig. 1, purity 99.9%) were kindly provided by Laboratory of Chemical Synthesis (Institute of Materia Medica, Chinese Academy of Medical Science). Methanol was of HPLC grade (Fisher, USA). All other chemicals were of analytical reagent grade. HPLC grade water was obtained using a Milli Q system (Millipore, MA, USA).

2.2. Instruments

The separation was carried out on an Agilent 1260 series HPLC system (Agilent, USA) with an injection of 10 μL sample onto a Zorbax SB-C18 column (100 mm × 2.1 mm, 3.5 μm, Agilent, USA). The temperature of the autosampler was set at 10 °C throughout the analysis. Mobile phase A was 0.1% (*v*/*v*) formic acid, mobile phase B was methanol with 0.1% (*v*/*v*) formic acid, and the flow rate was 0.2 mL/min with an operating temperature of 20 °C. The initial condition for the HPLC gradient was 70:30 (A:B). From 2.5 to 2.6 min, the mobile phase composition changed linearly to 5:95 (A:B). This condition was held until 4.6 min, followed by re-equilibration at 70:30 (A:B) until 9.0 min.

Mass spectrometric analysis was performed on the API 4000 triple quadruple mass spectrometer (AB SCIEX, USA) in positive electrospray ionization (ESI) mode. The optimized ion spray voltage and temperature were set at 5500 V and 500 °C. The collision gas (CAD) and curtain gas (CUR) were nitrogen delivered at 6 and 25 psi, while both ion source gas 1 (GS1) and ion source gas 2 (GS2) were set at 50 psi. The collision energy (CE) of H002, H002-P, H002-M and IS were 35, 25, 25 and 26 eV, respectively. The MS recordings were carried out in multiple reaction monitoring (MRM) mode with specific ion transitions of protonated precursor ion to product ion at *m*/*z* 367.2 → 350.1 for H002, *m*/*z* 447.1 → 320.1 for H002-P, *m*/*z* 383.3 → 366.4 for H002-M and *m*/*z* 381.2 → 364.2 for IS.

In addition, the MEK-7222K type blood cell analyzer (NIHON KOHDEN, Japan) was used to count PBL.

2.3. Preparation of standard and quality control samples

To prepare the stock solutions, H002, H002-M and H001 (IS) were dissolved in methanol at 1 mg/mL, while H002-P was 0.5 mg/mL, taking solubility into consideration. Working standards were prepared by dilution of stock solution in methanol to obtain the desired concentrations of 1, 2.5, 5, 10, 25, 100, 250, 375, 500 ng/mL for H002 and H002-M, 2.5, 5, 10, 25, 50, 100, 250, 375, 500 ng/mL for H002-P. The IS spiking solution was

prepared at 300 ng/mL by dilution of stock solution with methanol. All working solutions were stored at –20 °C until use.

Calibration standard samples were prepared by adding 20 μL of each corresponding working solution and IS working solution along with 120 μL acetonitrile into 100 μL of blank rat blood, giving the blood concentrations of 0.2, 0.5, 1, 2, 5, 20, 50, 75, 100 ng/mL for H002 and H002-M, 0.5, 1, 2, 5, 10, 20, 50, 75, 100 ng/mL for H002-P and 60 ng/mL for IS. The quality control (QC) samples (0.5, 25, 80 ng/mL for H002 and H002-M, 1.5, 25, 80 ng/mL for H002-P) were prepared in a manner similar to that used for preparation of the calibrator samples.

2.4. Preparation of samples

A 20 μL of IS working solution (300 ng/mL) and 180 μL precipitant (a mixture of 120 μL acetonitrile and 60 μL methanol) were added into 100 μL of rat blood. The mixture was vortex-mixed, followed by centrifugation at 14,000 rpm (Thermo Scientific Heraeus Fresco 21 centrifuge, ThermoFisher Scientific, Germany) for 5 min. A 10 μL supernatant sample was injected into LC–MS/MS system for analysis.

2.5. Method validation

2.5.1. Selectivity

Selectivity was evaluated by analyzing six independent blank rat blood samples to investigate the potential interferences at peak regions for H002, H002-P, H002-M and IS. Their chromatographic peaks were identified on the basis of their retention times and MRM responses.

2.5.2. Linearity, precision and accuracy

To determine the linearity of the calibration curve, a peak–area ratio (analyte/IS) versus the nominal concentration of analytes with weighted ($1/X^2$) least square linear regression was calculated. The calibration curve required a correlation coefficient (r^2) of 0.99 or better.

The precision and accuracy of the assay were detected by analysis of QC samples. Intra-day precision and accuracy was estimated by analyzing five QC samples at low, medium and high concentration levels within one day, while the inter-day precision and accuracy were performed on five consecutive days. The variability of determination was expressed as the relative standard deviation (RSD, %) and the accuracy was expressed as the relative error (RE, %). The criteria for acceptability included accuracy within ±15% RE from the nominal values and a precision within ±15% RSD, except for the lower limit of quantitation (LLOQ), which should not exceed ±20% of accuracy as well as precision.

2.5.3. Matrix effects

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes, which was evaluated by comparing the responses with deproteinized samples of six spiked rat blood QC samples to those of standard solutions at equivalent concentrations. Three QC concentrations were analyzed in five replicates.

2.5.4. Recovery

The recoveries of H002, H002-P and H002-M from blood were calculated by comparing the peak area of the extracted QC samples to that of analytes spiked to the blank sample extracts at the same concentration. The recoveries of two compounds in rat blood were examined at least five times.

2.5.5. Stability studies

Three freeze–thaws, long-term, short-term and post-preparative stabilities of H002, H002-P and H002-M in rat blood were tested using low-, mid- and high-quality control samples. Freeze–thaw stability was evaluated in up to three cycles, in which the samples were frozen and stored at –20 °C for 24 h, then thawed on ice. For short-term stability, fresh blood samples were kept on ice for 6 h before preparation. The stability of prepared blood samples was determined after keeping them at room temperature for 24 h or in the autosampler rack at 10 °C for 48 h. Long-term storage stability was acquired by assaying QC blood samples after storage at –20 °C for 30 days and 3 months. Dilution stability was evaluated using a spiked sample at a concentration higher than the upper limit of quantification samples (ULOQ). The result was obtained by comparing the back-calculated value of the sample after being diluted 10 times with the nominal value.

2.5.6. Carry-over

Carry-over was evaluated by triplicated measurements of a blank sample following the injection of the highest calibration standard. It was considered negligible if the measured peak area was no more than 20% of the area of LLOQ[16,17].

2.6. Pharmacokinetic study

To assess the applicability of present method, the pharmacokinetics of H002, H002-P and H002-M were investigated in male Sprague–Dawley (SD) rats after oral administration of H002 at 3, 10 and 30 mg/kg. The rats (180–220 g) were purchased from Beijing Vital River Experimental Animal Co., Ltd. All animal protocols were approved by Institute Animal Care and Welfare Committee. The rats were allowed free access to water and chow diet and fasted overnight with water before pharmacokinetic experiments.

The dosing solutions were prepared by suspending H002 in 0.5% hydroxypropyl methyl cellulose (HPMC). The rats were given H002 at dose of 3, 10 and 30 mg/kg *via* gavage. Blood samples were taken from each animal by orbital bleeding *via* capillary tubes at 5, 15, 30 min, 1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h after dosing. The blood samples were placed on ice and immediately treated as described in Section 2.4 and frozen at –20 °C until analyzed.

The pharmacokinetic parameters of H002, H002-P and H002-M were calculated using WinNonlin software version 6.3 based on non-compartmental analysis (Pharsight Corporation, Mountain View, USA). The blood concentrations of the tested compounds were expressed as mean ± SD.

2.7. Measurement of peripheral blood lymphocytes

Ten male SD rats were randomly divided into two groups. H002 (3 mg/kg) was given to rats by gavage, while the rats in control group received the same volume of vehicle. A 20 μL sample of blood was collected from the tail vein at 0, 2, 4, 8, 12, 24, 36, 48 and 72 h after dosing and the PBL counts were measured immediately ($n=5$)[18].

3. Results and discussion

3.1. Method development

Better specificity and sensitivity for the determination of H002, H002-P, H002-M and IS were obtained in positive mode compared

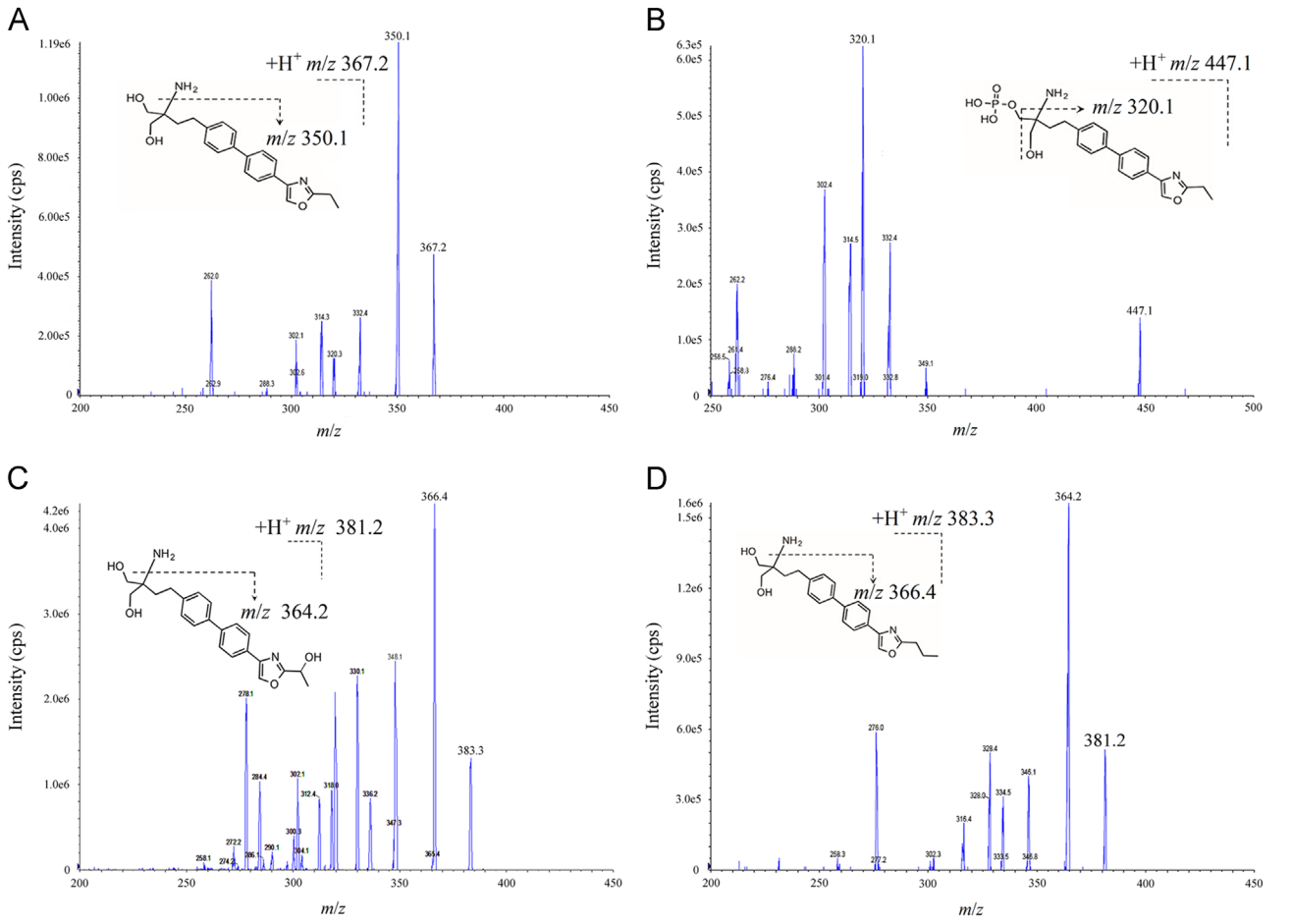

Figure 2 MS/MS spectra of (A) H002, (B) H002-P, (C) H002-M and (D) IS.

to negative mode. The positive ion electrospray mass spectra in full-scan Q1 mode showed a protonated molecular ion $[M+H]^+$ as the base peak, *m/z* 367.2 for H002, *m/z* 447.1 for H002-P, *m/z* 383.3 for H002-M and *m/z* 381.2 for IS. As shown in Fig. 2, the most abundant and stable product ions were at *m/z* 350.1, 320.1, 366.4 and 364.2 for H002, H002-P, H002-M and IS, respectively, after fragmentation in the collision cell. The mass spectrometric parameters, such as collision energy, spray voltage, and source temperature, were optimized for highest and most stable signal.

To achieve more satisfactory chromatographic behavior, different HPLC parameters including type of column material, mobile phase and appropriate flow rate were tested and compared. Zorbax SB-C18 column (3.5 μm, 100 mm × 2.1 mm) was adopted to obtain sharp peak shapes for each analyte. In this experiment, methanol/water containing 0.1% formic acid (*v/v*) at a flow rate of 0.2 mL/min was found to be suitable for the determination of electrospray responses of analytes.

3.2. *Method validation*

3.2.1. *Selectivity*

As shown in Fig. 3, there was no significant interference from rat blood observed at the retention times of the analytes and IS. The other peak in H002-M (Fig. 3 C) is speculated to be a relevant impurity peak, which could be an isomer produced in rat blood and needs further study. The retention times of H002, H002-P, H002-M and IS were 6.94, 7.16, 6.56 and 7.09 min, respectively.

3.2.2. *Linearity and range*

The liner relationships were found between peak area ratios and analyte concentrations within the range of 0.2–100 ng/mL for H002 and H002-M, and 0.5–100 ng/mL for H002-P. The regression equations obtained by least squared regression were $y=0.0157x+0.00123$ ($r^2=0.9980$, $n=5$) for H002, $y=0.0239x+0.00326$ ($r^2=0.9956$, $n=5$) for H002-P and $y=0.00623x+0.00101$ ($r^2=0.9953$, $n=5$) using weighing factor $1/X^2$. The observed deviations were within ±15% for all calibration concentrations. The LLOQ was set at 0.2 ng/mL for H002 and H002-M, 0.5 ng/mL for H002-P, which was appropriate for pharmacokinetic studies.

3.2.3. *Precision and accuracy*

The precision and accuracy of the method for H002, H002-P and H002-M were assessed by determining QC samples ($n=5$) at three concentrations and are summarized in Table 1. The intra- and inter-day precision of H002, H002-P and H002-M were within 11.76%, and the accuracy was found to be less than ±9.84%. The results indicate that the present method is reliable and reproducible for the quantitative determination of analytes.

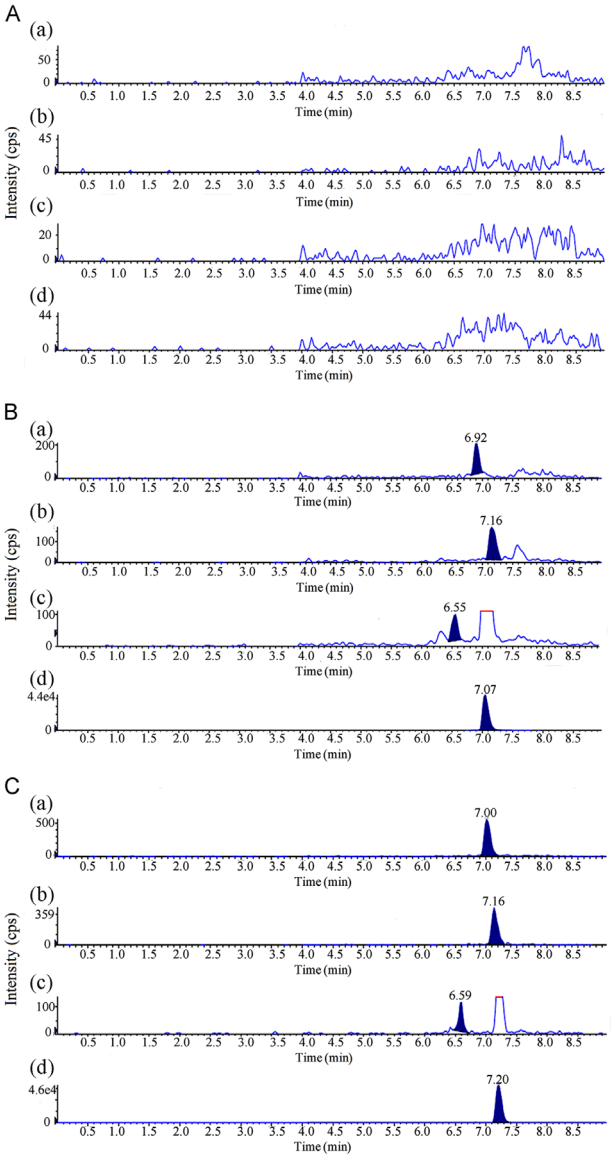

Figure 3 Typical MRM chromatograms of H002, H002-P, H002-M and IS. (A) Blank rat blood; (B) LLOQ sample and (C) rat blood sample at 15 min post dose of H002 (3 mg/kg) spiked with IS. (a) H002, (b) H002-P, (c) H002-M, (d) IS.

3.2.4. *Matrix effect*

The mean matrix effect values were 104.1%, 101.5% and 98.1% for H002, 97.6%, 102.1% and 99.9% for H002-P, and 95.0%, 101.9% and 99.9% for H002-M at low, medium and high concentration levels, respectively, suggesting that the matrix effect on the ionization was not significant under current conditions.

3.2.5. *Recovery and stability*

The recoveries from rat blood were 87.4%–106.0%, 94.8%–109.6% and 94.9%–113.3% for H002, H002-P and H002-M at three concentration levels.

All stability results are summarized in Table 2. The blood samples were shown to be stable after being placed on ice for 6 h, at room temperature for 24 h, stored at −20 °C for 30 days or 3 months, and through three freeze–thaw cycles or dilution. Furthermore, samples after treatment were stable at 10 °C in the autosampler for a period of 48 h, indicating that a large number of samples could be determined in each analytical run.

3.2.6. *Carry-over*

The analyte peak areas after triplicate injections of a blank blood sample and the highest calibration standard samples were less than 10% of LLOQ, demonstrating a negligible carry-over effect.

3.3. *Pharmacokinetic study and PBL counts*

The present method was applied to determine the blood concentration of H002, H002-P and H002-M in rats after oral administration of H002. According to previous pharmacological studies (unpublished data), oral administration of H002 at 3 mg/kg was found to be the effective dose in rats. Thus, we chose three doses (3, 10 and 30 mg/kg) to investigate whether H002 underwent linear pharmacokinetics in the range of 3–30 mg/kg. The mean concentration–time curve ($n=5$) is illustrated in Fig. 4 and the pharmacokinetic parameters are listed in Table 3. The C_{max} and T_{max} were obtained directly from the pharmacokinetic data while other parameters were calculated with WinNonlin software. The results show that the T_{max} of both H002-P and H002-M were 2–4 h

Table 1 Intra-day and inter-day accuracy and precision of measurement of H002, H002-P and H002-M in rat blood ($n=5$).

Sample	Nominal Conc. (ng/mL)	Observed Conc. (ng/mL)	Accuracy (RE, %)	Precision (RSD, %)
H002				
Intra-day	0.5	0.50±0.03	0	6.00
	25	27.10±0.68	8.40	2.51
	80	81.34±1.50	1.78	1.84
Inter-day	0.5	0.51±0.05	2.00	9.80
	25	27.28±0.78	9.12	2.86
	80	79.56±3.30	–0.55	4.15
H002-P				
Intra-day	1.5	1.43±0.09	–4.67	6.29
	25	24.14±0.73	–3.36	3.02
	80	84.00±3.67	5.00	4.37
Inter-day	1.5	1.44±0.13	–4.00	9.03
	25	25.28±0.63	1.12	2.49
	80	81.04±3.91	1.30	4.82
H002-M				
Intra-day	0.5	0.50±0.04	0	8.00
	25	22.54±0.55	–9.84	2.44
	80	72.76±1.04	–9.05	1.43
Inter-day	0.5	0.51±0.06	2.00	11.76
	25	23.08±1.30	–7.68	5.63
	80	73.64±4.43	–7.95	6.02

Table 2 Stability of H002, H002-P and H002-M in rat blood ($n=5$).

Condition	H002			H002-P			H002-M		
	Nominal Conc. (ng/mL)	Observed Conc. (ng/mL)	RE (%)	Nominal Conc. (ng/mL)	Observed Conc. (ng/mL)	RE (%)	Nominal Conc. (ng/mL)	Observed Conc. (ng/mL)	RE (%)
Store at 4 °C (6 h)	0.5	0.49±0.01	–2.00	1.5	1.62±0.07	8.00	0.5	0.49±0.02	–2.00
	25	25.74±0.23	2.96	25	23.10±0.43	–7.60	25	26.78±1.51	7.12
	80	77.72±1.86	–2.85	80	72.26±2.06	–9.68	80	79.66±2.34	–0.43
Room Temperature (24 h)	0.5	0.50±0.03	0.00	1.5	1.41±0.10	–6.00	0.5	0.49±0.04	–2.00
	25	26.24±0.61	4.96	25	23.58±0.59	–5.68	25	22.42±0.63	–10.32
	80	85.76±0.85	7.20	80	83.56±1.34	4.45	80	75.30±1.20	–5.88
Autosampler Rack at 10 °C (48 h)	0.5	0.54±0.03	8.00	1.5	1.53±0.05	2.00	0.5	0.47±0.04	–6.00
	25	26.48±0.90	5.92	25	25.78±1.09	3.12	25	22.30±1.12	–10.80
	80	84.08±1.00	5.10	80	81.52±4.02	0.67	80	71.14±1.44	–11.08
Store at –20 °C (7 d)	0.5	0.47±0.02	–6.00	1.5	1.51±0.15	2.40	0.5	0.45±0.01	–10.00
	25	25.18±0.46	0.72	25	25.60±0.45	–2.68	25	23.40±0.62	–6.40
	80	76.80±0.51	–4.00	80	77.86±2.53	2.39	80	82.88±3.55	3.60
Store at –20 °C (30 d)	0.5	0.51±0.04	2.00	1.5	1.45±0.09	–3.33	0.5	0.48±0.05	–4.00
	25	25.82±0.97	3.28	25	26.90±0.99	7.60	25	22.94±0.38	–8.24
	80	77.48±2.75	–3.15	80	77.62±2.88	–2.98	80	77.32±2.84	–3.35
Store at –20 °C (3 months)	0.5	0.47±0.02	–6.00	1.5	1.31±0.11	–12.67	0.5	0.46±0.03	–8.00
	25	24.70±0.75	–1.20	25	25.12±0.91	0.48	25	25.76±0.86	3.04
	80	75.36±0.55	–5.80	80	77.18±3.37	–3.53	80	82.22±3.40	2.78
Three freeze–thaw cycles	0.5	0.51±0.01	2.00	1.5	1.41±0.09	–6.00	0.5	0.48±0.01	–4.00
	25	27.18±0.33	8.72	25	24.70±0.41	–1.20	25	22.86±0.85	–8.56
	80	85.98±0.98	7.48	80	80.40±1.23	0.50	80	77.00±1.25	–3.75
Dilution stability (factor: 10)	80	73.09±0.94	–8.64	80	72.45±0.65	–9.44	80	83.49±0.94	4.36

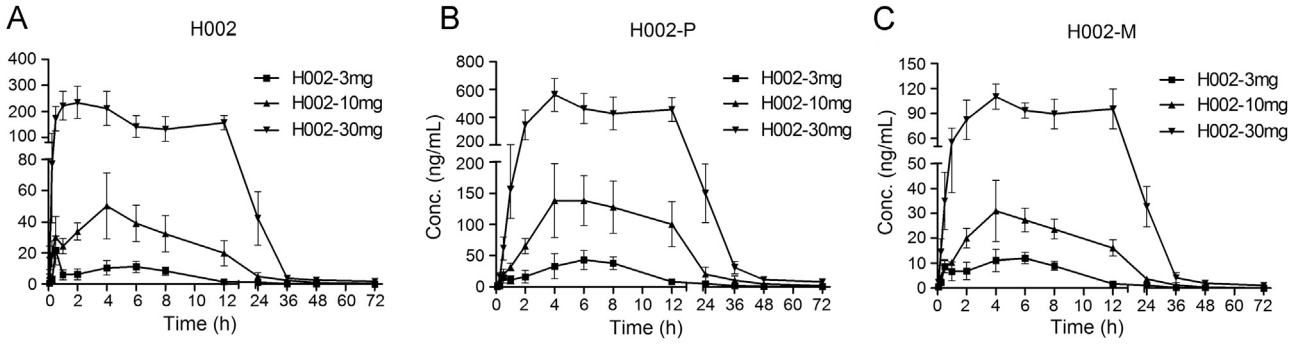

Figure 4 Mean blood concentration curves of (A) H002, (B) H002-P and (C) H002-M in rats after oral administration of H002 at 3, 10 and 30 mg/kg.

later than that of H002, while the C_{max} of H002-P and H002-M were 2.06–2.62- and 0.43–0.61-fold of the parent drug, respectively. The AUC_{0-t} and C_{max} values obtained from three oral dose levels showed a lack of linearity, along with the extension of $t_{1/2z}$ and MRT_{0-t} and reduction of V_z and CL_z with increased dosage, suggesting the saturation of absorption and elimination. The results indicate nonlinear pharmacokinetics for H002, H002-P and H002-M in rats from 3 to 30 mg/kg.

PBL counting is a specific and remarkable indicator for lymphocyte homing. As shown in Fig. 5, PBL counts decreased dramatically and were maintained for 8 h in rats after oral administration of H002 at 3 mg/kg, while high blood concentrations of the three analytes were observed over the same period. Compared with FTY720[8], a quicker recovery of lymphopenia after discontinuation was observed due to the rapid elimination of H002 *in vivo*, which is a desirable pharmacokinetic characteristic for $S1PR_1$-selective agonizts. For a better understanding and prediction of H002 concentration–effect relationship *in vivo*, a mechanism-based PK/PD model will be developed in future studies.

Table 3 Pharmacokinetic parameters of H002, H002-P and H002-M in male rats after a single oral dose of H002 at 3, 10 and 30 mg/kg ($n=5$).

Parameter	Unit	H002 (mg)		
		3	10	30
$t_{1/2z}$	h	13.42±3.16	10.41±1.84	18.37±1.19
T_{max}	h	1.20±1.57	4.00±1.41	2.00±1.22
C_{max}	ng/mL	22.20±8.16	55.30±17	267.60±56.99
AUC_{0-t}	(ng · h)/mL	125.79±24.38	630.27±181.38	3782.58±663.05
V_z	L/kg	457.53±67.33	245.31±50.79	215.78±43.99
CL_z	L/h/kg	24.42±5.16	16.74±4.28	8.10±1.34
MRT_{0-t}	h	11.17±2.57	11.75±2.4	14.63±3.09
Parameter	Unit	H002-P (mg)		
		3	10	30
$t_{1/2z}$	h	12.43±0.94	12.65±1.36	16.00±1.63
T_{max}	h	6.80±1.10	6.00±2.00	5.60±3.58
C_{max}	ng/mL	45.76±14.78	161.22±51.54	567.20±111.45
AUC_{0-t}	(ng · h)/mL	464.82±93.83	2348.96±733.10	10802.89±2551.27
V_z	L/kg	118.67±28.19	81.75±20.13	65.54±9.46
CL_z	L/h/kg	6.61±1.39	4.53±1.16	2.88±0.62
MRT_{0-t}	h	13.08±2.13	14.04±2.90	16.76±3.40
Parameter	Unit	H002-M (mg)		
		3	10	30
$t_{1/2z}$	h	5.95±1.12	7.07±0.62	16.62±1.39
T_{max}	h	4.80±1.10	6.80±3.35	6.00±3.46
C_{max}	ng/mL	13.06±2.84	33.50±10.56	114.46±18.14
AUC_{0-t}	(ng · h)/mL	127.40±9.10	427.18±33.89	2213.58±286.86
V_z	L/kg	330.56±60.12	239.14±21.36	202.54±39.94
CL_z	L/h/kg	23.61±1.74	23.50±1.81	13.71±1.68
MRT_{0-t}	h	10.03±1.69	11.32±1.82	16.19±2.18

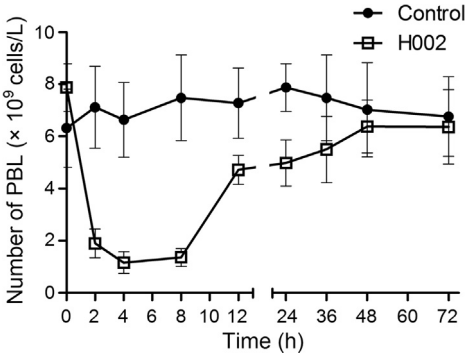

Figure 5 PBL counts–time curve in rats after oral administration of H002 (3 mg/kg) and vehicle.

4. Conclusions

In conclusion, the newly developed and validated LC–MS/MS method with protein precipitation provided an accurate, sensitive and simple assay for the simultaneous determination of H002, H002-P and H002-M, and was applied for the pharmacokinetic analysis of H002 in rats after oral administration. A preliminary relationship between the drug blood concentration and peripheral lympho-depletion was also observed.

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