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High-performance liquid chromatography–tandem mass spectrometry for the determination of pidotimod in human plasma and its application to a pharmacokinetic study

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ABSTRACT

A selective, rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed for the first time to determine pidotimod in human plasma and applied to a pharmacokinetic study. Diphenhydramine was used as the internal standard (I.S.). Sample pretreatment involved in one-step protein precipitation (PPT) with methanol of 0.1 mL plasma. The analysis was carried out on an Ultimate™ XB-C_8 column with mobile phase of methanol–water containing 0.5% formic acid (65:35, *v*/*v*). The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. Each plasma sample was chromatographed within 4.5 min. The linear calibration curves were obtained in the concentration range of 0.05–20.00 μg/mL ($r^2 \geq 0.99$) with the lower limit of quantification (LLOQ) of 0.0500 μg/mL. The intra- and inter-day precision (relative standard deviation, R.S.D.) values were below 15% and accuracy (relative error, R.E.) was from −5.1% to 3.9% at all quality control (QC) levels. The method was applicable to clinical pharmacokinetic study of pidotimod in healthy volunteers after oral administration.

1. Introduction

Pidotimod, (R)-3-[(S)-(5-oxo-2-pyrrolidinyl) carbonyl]-thiazolidine-4-carboxylic acid (Fig. 1A), is the first compound of a new class of biological response modifiers with a peptide-like structure, which can stimulate both the primary and acquired immune response to virus and bacteria [1]. Studies demonstrated that pidotimod itself does not have antibacterial activity, but combined with antibacterial agents can be effective in improving clinical symptoms of patients, promoting recovery and shortening hospital stay. To date, however, pharmacokinetic studies of pidotimod are still seldom reported. Therefore, the development of a sensitive and specific method to determine pidotimod in human plasma is necessary and valuable.

Several analytical methods have been applied to the quantification of pidotimod in biological fluids, including HPLC-UV [2–4] and HPLC–MS [5]. But the low sensitivity (LLOQ higher than 0.10 μg/mL) [3,4], long analysis time (longer than 8 min) [5] or large volumes of plasma samples (larger than 0.5 mL) [3,4] may not meet the requirement of desired sample throughput, speed and sensitivity in pharmacokinetic and clinical studies of pidotimod. To our knowledge, the quantification of pidotimod in human plasma using tandem mass spectrometry has not been reported. Tandem MS with multiple reaction monitoring (MRM) mode usually has higher signal-to-noise ratio and then higher sensitivity [6,7]. This paper describes for the first time a high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method, which facilitates the determination of pidotimod with good accuracy at low drug concentration in human plasma. The method was fully validated and applied to the pharmacokinetic study in healthy volunteers after oral administration of 800 mg pidotimod in tablets.

2. Experimental

2.1. Reagents and chemicals

Reference standard of pidotimod (99.6% purity, Fig. 1A) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Diphenhydramine (I.S., 99.5% purity, Fig. 1B) was kindly provided by Medicinal Chemistry Department of Shenyang Pharmaceutical University. Methanol of HPLC grade was obtained from Tedia (Fairfield, OH, USA). Formic acid (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

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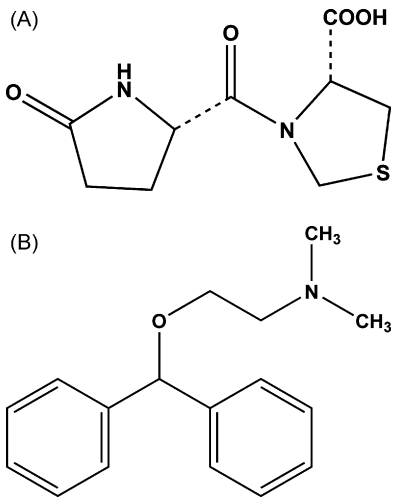

Fig. 1. Chemical structures of pidotimod (A) and diphenhydramine (I.S.) (B).

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The chromatography was performed on an ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) with cooling autosampler. An Ultimate™ XB-C_8 column (50 mm × 4.6 mm i.d., 5 μm) was employed for the separation at ambient temperature. The mobile phase was composed of methanol–water containing 0.5% formic acid (65:35, *v*/*v*). The flow rate was set at 0.20 mL/min. The autosampler temperature was kept at 4 °C and 10 μL of sample solution was injected with partial loop mode.

2.2.2. Mass spectrometry

Mass spectrometric detection was carried out on a Micromass® Quattro micro™ API triple–quadrupole tandem mass spectrometer (Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m*/*z* 245.0 → 133.7 for pidotimod and *m*/*z* 256.0 → 166.8 for diphenhydramine (I.S.) respectively, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 2.5 kV, cone voltage 18 V, source temperature 110 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 400 and 50 L/h respectively. Argon was used as the collision gas at a pressure of approximately 0.255 Pa. The optimized collision energy for the analyte and I.S. was 18 and 10 eV respectively. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Stock standard solutions of pidotimod and diphenhydramine were prepared in methanol at the concentration of 400 and 105 μg/mL respectively. The internal standard (I.S.) solution was prepared by dilution with methanol to 52.5 ng/mL. And pidotimod stock solution was serially diluted with methanol to provide working standard solutions at desired concentrations. In addition, appropriate amount of pidotimod was dissolved in methanol to give a final concentration of 200 μg/mL for the preparation of quality control (QC) samples. All the solutions were stored at 4 °C and brought to room temperature before use.

Calibration standards were prepared by evaporating 50 μL of working standard solutions to dryness and then fully mixing with 100 μL of blank plasma. The effective concentrations in standard plasma samples were 0.0500, 0.150, 0.500, 1.00, 2.50, 5.00, 10.0, and 20.0 μg/mL. One calibration curve was constructed on each analysis day using freshly prepared calibration standards. The QC samples were prepared in bulk with the same procedure as for standard samples and aliquots were stored at −20 °C. The LLOQ, low, mid and high concentrations of QC samples were 0.0500, 0.100, 2.00 and 16.0 μg/mL. The standards and quality controls were extracted on each analysis day with the same procedures for plasma samples as described below.

2.4. Plasma sample preparation

Fifty microlitres of I.S. solution were pipetted into 1.5 mL polypropylene micro-centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was vertex-mixed with 100 μL of plasma for 30 s. And then 300 μL of methanol was added to each tube. The mixture was vertex-mixed for 60 s and centrifuged at 11 200 × *g* for 10 min. The supernatant (300 μL) was transferred to an autosampler vial and an aliquot of 10 μL was injected into the HPLC–MS/MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery and stability according to FDA guidance for validation of bioanalytical methods [8]. Validation runs were conducted on three consecutive days. The peak area ratios of pidotimod to the I.S. of QC samples were interpolated from the calibration curve on the same day to give the concentration of pidotimod. The results from QC samples in three runs were used to evaluate the precision and accuracy of the method developed.

2.5.1. Selectivity

The selectivity was investigated by comparing chromatograms of six different batches of blank plasma from six subjects to those of corresponding standard plasma samples spiked with pidotimod and I.S. (52.5 ng/mL) and plasma sample after oral dose of pidotimod tablets.

2.5.2. Linearity and lower limit of quantification

Calibration curves were constructed by assaying standard plasma samples at eight concentrations in the range of 0.0500–20.0 μg/mL with weighted ($1/x^2$) least squares linear regression. According to USP guidance [9], the limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated. The value of LOD was calculated as the analyte concentration which gives rise to peak whose height is 3 times the baseline noise. According to FDA guidance, the LLOQ is defined as the lowest amount of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy, which are an R.S.D. of 20% and an R.E. of 80–120% in this assay.

2.5.3. Precision and accuracy

The intra-day precision and accuracy were evaluated by using replicate analysis of QC samples of pidotimod on the same day. The validation run consisted of two sets of calibration standards and six replicates of LLOQ and QC samples at three concentrations. For determining the inter-day accuracy and precision, analysis of three batches of QC samples was performed on three consecutive days. The precision was expressed as the relative standard deviation (R.S.D.) and the accuracy as the relative error (R.E.).

2.5.4. Extraction recovery and matrix effect

The extraction recovery was determined by dividing the peak areas of pidotimod added into blank plasma and extracted using PPT procedure with those obtained from the compound spiked into equivalent volume of post-extraction supernatant. This procedure was repeated for five replicates at three QC concentration levels of 0.100, 2.00 and 16.0 μg/mL. The matrix effect was measured by comparing the peak response of sample spiked post-extraction (A) with that of pure standard solution containing equivalent amount of the compound (B). The ratio $(A/B \times 100)\%$ was used to evaluate the matrix effect. The extraction recovery and matrix effect of I.S. were also evaluated using the same procedure.

2.5.5. Stability

The stability of pidotimod in human plasma was assessed by analyzing three replicates of low, mid and high QC samples under different temperature and time conditions. Freeze–thaw stability was performed by subjecting unextracted QC samples to three freeze (−20 °C)–thaw (room temperature) cycles. QC samples were stored at −20 °C for 30 days and at ambient temperature for 4 h to determine long-term and short-term stability respectively. Post-preparative stability was studied by analyzing the extracted QC samples kept in the autosampler at 4 °C for 12 h. All stability testing QC samples were determined by using calibration curve of freshly prepared standards. The concentrations obtained were compared with the nominal values.

2.6. Application to pharmacokinetic study

The method was applied to determine the plasma concentrations of pidotimod from a clinical trial in which 20 healthy male volunteers received two pidotimod tablets (containing 400 mg pidotimod each). The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected into sodium heparin-containing tubes before and at 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 9.0, and 12.0 h post-dosing. The plasma was separated by centrifugation and stored at −20 °C until analyzed.

The maximum plasma concentrations (C_{max}) and their times (T_{max}) were noted directly from the measured data. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Selection of internal standard

According to FDA guidance, an internal standard in the analysis of biological sample could be a structurally similar analog of analyte or a stable labeled compound [8]. Deuterated standard would be a preferred internal standard in HPLC–MS assay; however, it is not always commercially available. Therefore, a compound that has structure, extraction recovery, chromatographic and mass spectrometric behavior similar to the analyte may be considered. In our case, sulbactam, diphenhydramine and phenacetin though belonging to different classes of compounds but with some structural similarity to the analyte were tested as the internal standard. Finally, diphenhydramine was chosen as I.S. due to its similarity to pidotimod in retention, ionization and extraction efficiency.

3.2. Optimization of mass spectrometry

HPLC–MS/MS operation parameters were carefully optimized for the determination of pidotimod. A standard solution (1 μg/mL) of pidotimod and diphenhydramine was directly infused along with the mobile phase into the mass spectrometer with ESI as the ionization source. And the mass spectrometer was tuned in both positive and negative ionization modes for pidotimod containing secondary amino and carboxy groups. The response observed in positive ionization mode was higher than that in negative ionization mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ at m/z 245.0 and 256.0 for pidotimod and diphenhydramine respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecule of pidotimod. The product ion scan spectra showed high abundance fragment ions at m/z 133.7 and 166.8 for pidotimod and I.S. respectively. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of m/z 133.7 for pidotimod. The ion transitions of m/z 245.0 → 133.7 for pidotimod and m/z 256.0 → 166.8 for I.S. were chosen for MRM.

3.3. Optimization of chromatography

Chromatographic conditions were optimized to obtain high sensitivity and sample throughput. The mobile phase systems of acetonitrile–water and methanol–water in various proportions were tested. The signal-to-noise (S/N) ratio of pidotimod was obviously higher with methanol–water as the mobile phase than that with acetonitrile–water. Methanol proportion in the mobile phase from 60% to 90% was considered in the following experiment. In view of the response of pidotimod, retention times and peak shapes of both pidotimod and I.S., 65% methanol was the best.

The ionization of pidotimod and diphenhydramine was increased by adding the additive in the mobile phase. Therefore, formic acid and ammonium acetate were added into the mobile phase to improve the response. The response of pidotimod was distinctly increased by adding formic acid. The effect of formic acid of 0.3%, 0.5% and 1.0% in aqueous phase on the response and peak shape of pidotimod was investigated and 0.5% formic acid was found to be the best. Finally, methanol–water containing 0.5% formic acid (65:35, v/v) was adopted as the mobile phase.

It was difficult for pidotimod to be retained on C_{18} column due to its strong polarity. The use of a C_8 column benefited the retention of the analyte and the separation from endogenous interference. The short column provided a run time as short as 4.5 min per sample, which was shorter than reported ones [2–5]. Although the chromatography was performed on an HPLC column (not under UPLC conditions), the small dead volume of Acquity system still profited the separation efficiency and run time.

Two channels were used for recording the response, channel 1 for pidotimod at retention time of 3.8 min, and channel 2 for the I.S. at retention time of 4.0 min. No interference was observed for both pidotimod and I.S. (Fig. 2).

3.4. Selection of extraction method

As pidotimod is a hydrophilic compound, protein precipitation was chosen as the sample preparation method. Several protein precipitants such as ethanol, methanol and acetonitrile were investigated. Both methanol and acetonitrile could be taken as the protein precipitant for they provided equivalent extraction

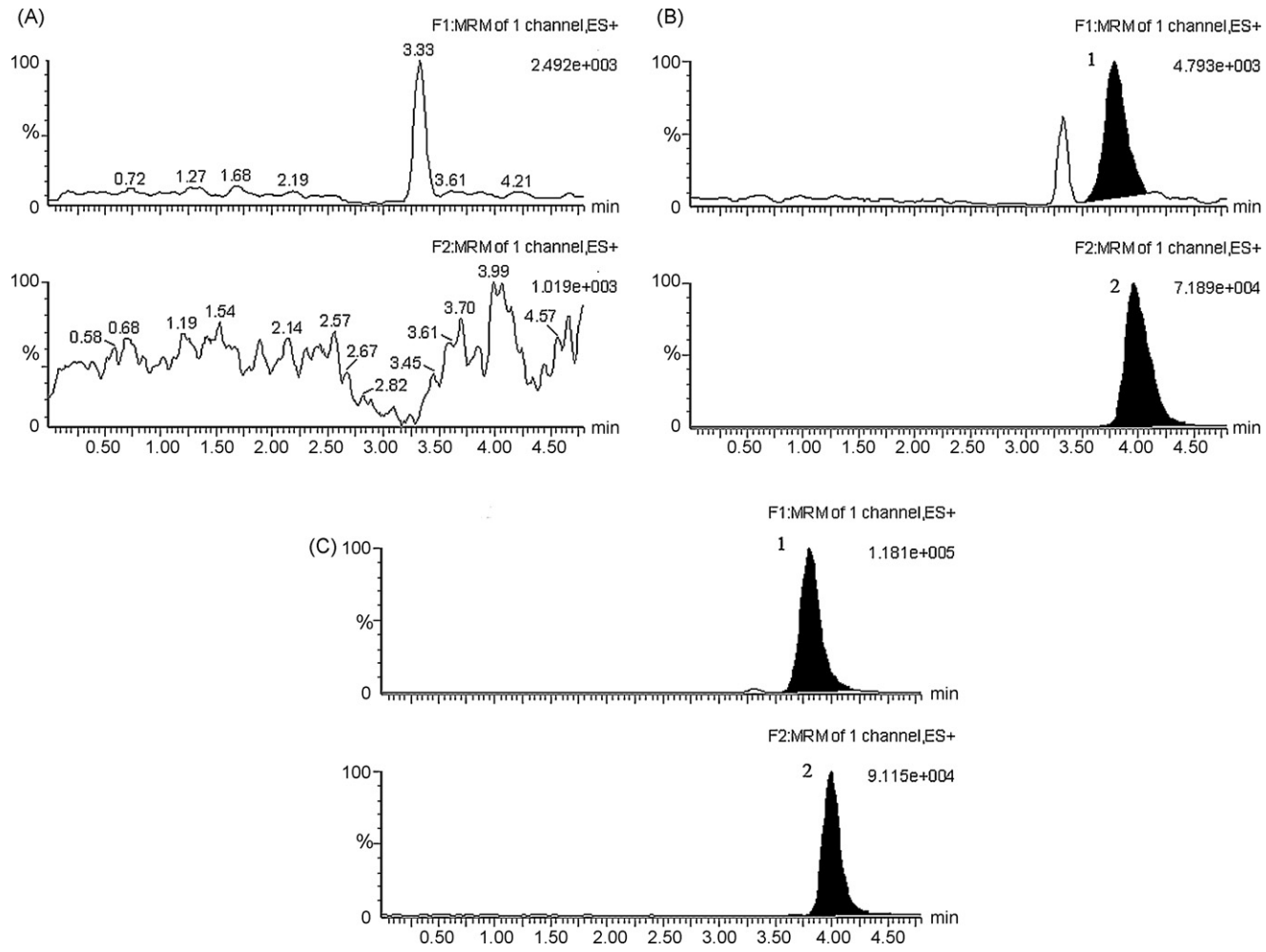

Fig. 2. Representative MRM chromatograms of pidotimod (peak 1, channel 1) and diphenhydramine (peak 2, channel 2) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with pidotimod at the LLOQ of 0.0500 μg/mL and diphenhydramine (52.5 ng/mL); (C) a plasma sample from a volunteer 0.5 h after oral administration of pidotimod. The retention times of pidotimod and diphenhydramine were 3.8 and 4.0 min respectively.

recovery. Methanol was chosen as the precipitant for its better compatibility with mobile phase.

3.5. Method validation

3.5.1. Selectivity

Comparing the chromatograms of six batches of blank plasma with the spiked plasma demonstrated a good selectivity of the method. As shown in Fig. 2A, the compound of interest was well separated from endogenous interference. Carry-over was eliminated by rinsing system, which was demonstrated by analyzing blank samples immediately following the samples at the highest concentration.

3.5.2. Linearity and LLOQ

The standard calibration curves for pidotimod were linear over the concentration range of 0.0500–20.0 μg/mL ($r^2 \geq 0.99$), with the value of LOD (0.0200 μg/mL). A typical regression equation for the calibration curves was $y = 2.70 \times 10^{-1}x + 5.59 \times 10^{-4}$, $r = 0.9946$, where y is the peak area ratio of pidotimod to I.S., and x is the concentration of pidotimod in plasma. The data of the linearity parameters of the method during the method validation are given in Table 1.

The lower limit of quantification (LLOQ) for pidotimod was 0.0500 μg/mL in plasma with precision (R.S.D.) below 20% and accuracy (R.E.) within ±20% (Table 2), which was lower than that reported in the literature [2–5]. A corresponding chromatogram is given in Fig. 2B. With present LLOQ of 0.0500 μg/mL, the pidotimod concentration can be determined in plasma samples until 12 h after a single oral dose of 800 mg pidotimod, which is sensitive enough to investigate the pharmacokinetic behavior of pidotimod in human.

3.5.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy of the method are given in Table 2. The intra- and inter-day R.S.D.s were not more than 7.1% and 14.9%, and R.E.s were from −5.5% to 3.9% at three QC levels, indicating acceptable precision and accuracy of the present method.

3.5.4. Extraction recovery and matrix effect

The extract recoveries of pidotimod from human plasma were 80.3 ± 3.2%, 90.9 ± 4.1%, and 82.7 ± 2.7% at concentrations of 0.100,

Table 1
Linearity parameters of the method in the range of 0.0500–20.0 μg/mL during method validation.

Run	Intercept ($\times 10^{-4}$)	Slope ($\times 10^{-1}$)	Regression
1	5.59	2.70	0.9946
2	5.90	2.22	0.9975
3	6.25	2.41	0.9976
Mean	5.91	2.44	0.9966
S.D.	0.33	0.24	0.0017

Table 2
Precision and accuracy for the determination of pidotimod in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, three days).

	Added C (μg/mL)	Found C (μg/mL)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Accuracy R.E. (%)
LLOQ	0.0500	0.0473 ± 0.0030	6.6	2.5	−5.5
Low QC	0.100	0.0959 ± 0.0076	7.1	12.2	−4.1
Mid QC	2.00	2.08 ± 0.14	4.8	14.9	3.9
High QC	16.0	16.3 ± 1.2	6.5	11.7	1.6

Table 3
Stability of pidotimod in human plasma at three QC levels ($n = 3$).

Stability	Mean ± S.D.		
	0.100 (μg/mL)	2.00 (μg/mL)	16.0 (μg/mL)
Short-term stability	0.101 ± 0.007	2.06 ± 0.23	16.8 ± 2.0
Long-term stability	0.103 ± 0.006	2.20 ± 0.09	16.2 ± 2.2
Freeze–thaw stability	0.0971 ± 0.0081	2.18 ± 0.12	16.5 ± 1.9
Post-preparative stability	0.103 ± 0.006	2.11 ± 0.09	17.4 ± 0.8

2.00, and 16.0 μg/mL respectively. The mean extraction recovery of I.S. was 93.9 ± 1.5%. Thus, the consistency in recoveries of pidotimod and I.S. supported the extraction procedure for its application to routine sample analysis.

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus, the evaluation of matrix effect from the influence of co-eluting components on analyte ionization is necessary for an HPLC–MS/MS method. All the ratios defined as in Section 2 were between 85% and 115%. No significant matrix effect for pidotimod and diphenhydramine was observed indicating that no co-eluting substance influenced the ionization of the analytes and I.S.

3.5.5. Stability of samples

The results from all stability tests are presented in Table 3, which indicated a good stability of pidotimod in plasma stored at room temperature for 4 h, at −20 °C for 30 days and during three freeze–thaw cycles, and in prepared samples at 4 °C for 12 h. The method is therefore proved to be applicable for routine analysis.

3.6. Pharmacokinetic application

This validated HPLC–MS/MS method was successfully applied to the pharmacokinetic study of pidotimod in healthy male volunteers after oral administration. Mean plasma concentration–time curve of pidotimod in single dose study is shown in Fig. 3.

After administration of a single dose of 800 mg pidotimod, the C_{max} and T_{max} were 5.57 ± 2.57 μg/mL and 1.87 ± 0.23 h respectively. Plasma concentration declined with a $t_{1/2}$ of 1.82 ± 0.27 h. The

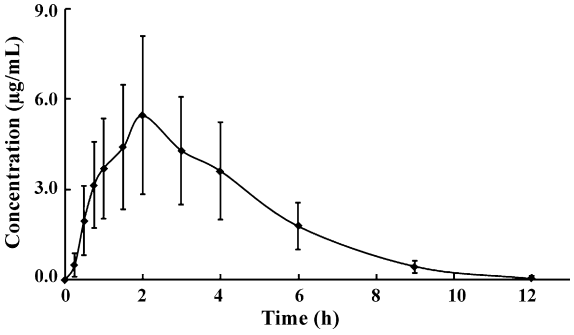

Fig. 3. Mean plasma concentration–time curve of pidotimod in male volunteers after a single oral dose of pidotimod.

AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 24.0 ± 9.6 μg h/mL and 25.1 ± 9.6 μg h/mL respectively. These pharmacokinetic parameters were in accordance with those reported in the literatures [3–5,10], indicating the applicability of this method to the pharmacokinetic study of pidotimod.

4. Conclusion

A sensitive, selective and rapid HPLC–MS/MS method for the determination of pidotimod in human plasma is described for the first time. Comparing with the analytical methods reported in the literatures, the method offered superior sensitivity with an LLOQ of 0.0500 μg/mL, satisfactory selectivity and short run time of 4.5 min. The method has been successfully applied to the pharmacokinetic study of pidotimod given in tablet to healthy volunteers.

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