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FULL SHORT COMMUNICATION

HILIC-MS-MS for the Quantification of Pidotimod in Human Plasma

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Abstract A method for fast, sensitive, and specific hydrophilic interaction chromatography combined with tandem mass spectrometry (HILIC-MS/MS) was developed for the first time to determine the level of pidotimod in human plasma. With rosiglitazone as internal standard, analysis was carried out on a HILIC column (150 mm × 2.1 mm, 3.5 μm) using a mobile phase consisting of methanol:0.2% formic acid (60:40, *v*/*v*). Detection was carried out by tandem mass spectrometry using electrospray ionization (ESI). Linear calibration curves were obtained in the concentration range of 11.2–1.12 × 10^4 ng mL^{-1} for pidotimod, with a lower limit of quantification of 11.2 ng mL^{-1}. The intra- and inter-day precision values were high, with standard deviations lower than 15%, and the accuracy, in terms of relative error, ranged from −10.5 to 9.4% at all quality control (QC) levels.

Keywords HILIC–MS/MS · ESI · Human plasma · Pidotimod · Rosiglitazone

Introduction

Pidotimod (*R*)-3-[(*S*)-(5-oxo-2-pyrrolidinyl)carbonyl]-thiazolidine-4-carboxylic acid (Fig. 1a) is an immunopotentiating agent. It not only can stimulate nonspecific immune reactions, but also can stimulate specific immune reactions. Previous studies have shown that pidotimod by itself does not have antibacterial activity, but in combination with antibacterial agents, it can be effective in the treatment of people infected with bacteria and viruses [1]. Pidotimod is often used in the treatment of repeated infections of the respiratory, urogenital, and ear, nose, and throat systems. Pidotimod therapy is a reliable, simple, and safe approach to treat children with recurrent respiratory infections, and it can reduce the frequency of such infections by improving the cilia of the respiratory epithelium. It has few adverse effects and demonstrates good safety and tolerability [2]. However, reports on pharmacokinetic study of pidotimod are few. Therefore, it is desirable to develop a sensitive and selective method for the analysis of pidotimod in human plasma.

Several HPLC-UV methods [1–5] have been developed for the quantification of pidotimod in biological samples. However, these methods involve both low sensitivity (lower limit of quantification, LLOQ higher than 0.1 μg mL^{-1}) [1, 4, 5] and a long analysis time (longer than 8 min) [1–5]. Furthermore, the volume of plasma used in these methods is large (larger than 0.5 mL) [1–4] and may not meet the requirements for high throughput, speed, and sensitivity in biosample analysis. Mass spectrometry (MS) has been widely used for the determination of compounds in biological samples because of its high sensitivity. Tandem MS (MS/MS) has often been applied because of its specificity. Electrospray ionization (ESI) is the most common method of sample introduction; as a 'soft' ionization technique, ESI results in less fragmentation of the analytes, so that spectral data are often less complicated [6]. There is only one report with reference to the determination of pidotimod using HPLC in association with

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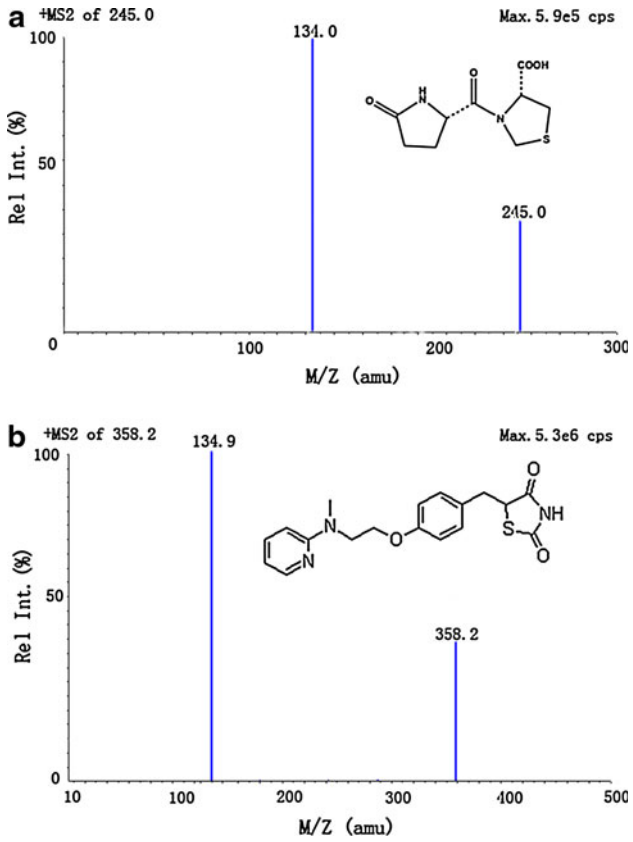

Fig. 1 **a** Chemical structures and product ion spectra of pidotimod. **b** Chemical structures and product ion spectra of rosiglitazone

tandem mass spectrometry (HPLC-MS/MS) [7]. The LLOQ in this method was 50 ng mL^{-1}, and the analysis time was 4.5 min. All these HPLC and HPLC–MS/MS methods use a C_{18} or C_8 column to separate pidotimod. However, due to the highly polar characteristic of pidotimod, C18 and C8 columns may not separate pidotimod well from the matrix. The matrix of plasma is very complex, especially when using protein precipitation. A column suitable for polar compounds is considered useful for a satisfactory separation of pidotimod and the matrix.

This article describes, for the first time, a hydrophilic interaction chromatography procedure combined with tandem MS (HILIC-MS/MS) for the determination of pidotimod in human plasma. HILIC is a type of liquid chromatography that allows high-resolution separation of highly polar compounds [8]. The LLOQ in this method was found to be 11.2 ng mL^{-1}, much lower than reported in literature (higher than 50 ng mL^{-1}) [1, 4, 5, 7]. The total analysis time of 3.5 min is shorter than times reported in the literature (longer than 4.5 min) [1–5, 7]. The volumes of plasma used were 0.5 [1, 4] or 1 mL [2, 3] larger than in this method (0.2 mL). This method can be conveniently applied to the pharmaceutical study of pidotimod in human plasma.

Experimental

Chemicals and Reagents

Pidotimod (reference standard 99.6% purity) and rosiglitazone (99.3% purity) were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile, methanol, and formic acid (LC grade) were purchased form Dikma (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22-μm membrane filter before use.

Instruments and Conditions

The chromatography was carried out on an Agilent 1200 system (Agilent, Santa Clara, CA, USA) with an autosampler and column oven enabling temperature control of the analytical column. An Inertsil HILIC column (150 mm × 3.0 mm, 3.5 μm) was used. The column was maintained at room temperature. The mobile phase consisted of methanol:0.2% formic acid (60:40, *v*/*v*) at an isocratic flow rate of 0.35 mL min^{-1}. The injection volume was 5 μL.

Detection was carried out on a Sciex API 4000 Qtrap MS system (Applied Biosystems Inc., Foster City, CA, USA), equipped with a Turbo Ionspray interface. The mass spectral settings for operating in the positive-ion mode (ESI+) were: ion source voltage: 5,000 V; ion source temperature: 600 °C; collision gas (N_2): medium; curtain gas: 30 psi; nebulizer gas: 60 psi; auxiliary gas: 60 psi. Quantification was carried out using multiple reaction monitoring (MRM) of transitions of *m/z* 245.0 → *m/z* 134.0 for pidotimod and *m/z* 358.2 → *m/z* 134.9 for rosiglitazone (Fig. 1). Data acquisition and processing were conducted with the Analyst 1.5 software (Applied Biosystems).

Preparation of Standards and Quality Control Samples

Standard stock solutions of pidotimod and rosiglitazone were prepared in a methanol:water mixture (1:1, *v*/*v*) at concentrations of 1.12 mg mL^{-1} and 155 μg mL^{-1}, respectively. The solutions were serially diluted with a methanol:water mixture (1:1, *v*/*v*) to provide working standard solutions of desired concentrations. All the solutions were stored at 4 °C.

Calibration standards were prepared by spiking 0.2 mL of blank human plasma with 50 μL of pidotimod working standard solutions and 50 μL of the internal standard (IS, rosiglitazone). The concentrations in standard plasma samples were 11.2, 22.4, 56.0, 224, 560, 2.24 × 10^3, and 1.12 × 10^4 ng mL^{-1} for pidotimod. The calibration curve was constructed on each day of analysis using freshly prepared calibration standards. The quality control (QC) samples were prepared with blank plasma at LLOQ, low,

medium, and high concentrations of 11.2, 28.0, 1.12×10^3, and 8.96×10^3 ng mL^{-1} for pidotimod. The standards and QC samples were extracted on each day of analysis by the procedure used for plasma samples as follows.

Plasma Sample Preparation

To a 0.2-mL aliquot of plasma sample in a 1.5-mL centrifuge tube, 50 μL of water:methanol mixture (1:1, *v/v*), 50 μL of IS (93.0 ng mL^{-1}), and 400 μL of methanol were added. The mixture was vortexed thoroughly for 1 min and then centrifuged at 13,000 rpm for 10 min. The supernatant was directly injected into the HILIC-MS/MS system.

Method Validation

Validation runs were conducted on 3 consecutive days. Each validation run consisted of a minimum of one set of calibration standards and six replicates of LLOQ and QC plasma samples at three concentrations. The results from the LLOQ and QC plasma samples in these three runs were used to evaluate the precision and accuracy of the method developed.

Selectivity

Selectivity was studied by comparing the chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with pidotimod and rosiglitazone (93.0 ng mL^{-1}).

Linearity and LLOQ

Calibration curves were prepared by assaying standard plasma samples at seven concentrations of pidotimod in the range of 11.2 to 1.12×10^4 ng mL^{-1}. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of pidotimod to rosiglitazone versus the nominal concentration (x) of pidotimod. The calibration curves were constructed by weighted ($1/x^2$) least square linear regression.

The LLOQ, defined as the lowest concentration on the calibration curve, was validated using an LLOQ sample, for which an acceptable accuracy (relative error, RE within ±20%) and a precision (relative standard deviation, RSD below 20%) were obtained.

Precision and Accuracy

For determining the intra-day accuracy and precision, a replicate analysis of the QC samples of pidotimod was carried out on the same day. The run consisted of a calibration curve and six replicates each of LLOQ, low, medium, and high concentration QC samples. The inter-day accuracy and precision were assessed by the analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD) and the accuracy as the relative error (RE).

Extraction Recovery and Matrix Effect

The recovery was calculated by comparing the peak area of the pidotimod added into blank plasma and extracted using the protein precipitation procedure with those obtained from the compound spiked into the postextraction supernatant at three QC concentration levels. The matrix effect was measured by comparing the peak response of the sample spiked postextraction (A) with that of the standard solution containing equivalent amounts of the compound (B). The ratio ($A/B \times 100$) % was used to evaluate the matrix effect. The extraction recovery and matrix effect of IS were also evaluated using the same method.

Stability

The stability of pidotimod in human plasma was assessed by analyzing five replicates of low, medium, and high concentration QC samples under different temperatures and timing conditions. Freeze-thaw stability was evaluated by subjecting the unextracted QC samples to three freeze (−20 °C)-thaw (room temperature) cycles. The QC samples were stored at −20 °C for 25 days and at ambient temperature for 4 h to determine their long-term and short-term stability, respectively. Post-preparative stability was studied by analyzing the extracted QC samples stored in the autosampler at 4 °C for 8 h. All the stability-tested QC samples were estimated using calibration curves of freshly prepared standards. The concentrations obtained were compared with the nominal values. The stability of stock solution of pidotimod was evaluated at 25 °C for 4 h and 4 °C for 30 days.

Application to Pharmacokinetic Study

The method was successfully applied to determine the pharmacokinetics of pidotimod granules in 20 healthy Chinese volunteers. Every subject was orally administered 800 mg of pidotimod. The pharmacokinetic study was approved by the local ethics committee, and 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 before and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, and 14 h post-dosing. Samples were centrifuged, and the plasma was separated and stored at −20 °C until analysis.

The maximum concentration in plasma (C_{max}) and the time required to achieve this level were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semilog plot of the 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 ($AUC_{0\text{-}t}$) until 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 using the expression: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

Results and Discussion

Selection of IS

The best IS for an LC-MS assay is a deuterated form of the analyte. In our laboratory, no deuterated pidotimod was available. Therefore, a compound structurally or chemically similar to the analyte was considered. In LC-MS/MS, the IS should also have chromatographic and mass spectrometric profiles similar to those of the analyte and should mimic the analyte in any sample preparation step. Rosiglitazone was chosen as the IS for the assay because of its similarity in terms of structure, retention time, and ionization to pidotimod.

Chromatography and Mass Spectrometry

Due to the highly polar characteristics of pidotimod, simple chromatographic methods cannot be applied for separation: pidotimod is not retained on conventional C_{18}-bonded silica columns without an ion-pairing reagent. Ion pair chromatography has been described to prolong the retention of highly polar compounds. However, ESI-MS detection of ion pairs is not ideal because the sensitivity of mass spectrometry is reduced (due to suppression of ionization). An alternative technique for the separation of hydrophilic compounds is HILIC, wherein analyte retention is believed to be caused by partitioning of the analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent. The use of water as the strongly eluting solvent gives HILIC a number of advantages over conventional normal phase chromatography (NPC). NP eluents are also nonpolar (often based on hexane), and polar analytes usually have a low solubility in these eluents. The interfacing with ESI-MS is also a problem with NPC, because ionization is not easily achieved in totally organic, nonpolar eluents. The elution order in HILIC is more or less the opposite of that seen in reversed-phase (RP) separations, which means that HILIC works best for solutes that are difficult to separate by RP [8]. Therefore, HILIC was chosen for the separation.

Ionization of the analyte is affected by the composition of the mobile phase. Ammonium acetate and formic acid were tried to complement the ionic strength. Formic acid was better than ammonium acetate in improving the response of pidotimod. The effect of formic acid (0.1, 0.2, and 0.3% in the aqueous phase) on the response of pidotimod was investigated, and 0.2% was found to be the best concentration. Methanol was better than acetonitrile in improving the shapes of the pidotimod peaks. Hence, a mixture of water with 0.2% formic acid-methanol was finally adopted as the mobile phase.

The LLOQ for pidotimod was 11.2 ng mL^{-1}. Due to the small injection volume (5 μL), the on-column sensitivity (the quantity of drug injected onto the column per injection) in our study was 56 pg, which was much lower than those reported in literature, which were higher than 100 ng mL^{-1} at injection volumes of 20 [1, 4] or 10 μL [5] using HPLC-UV and much lower than the reported value of 50 ng mL^{-1} with an injection volume of 10 μL using HPLC-MS/MS [7]. All the methods in the literature use C_{18} or C_8 columns to analyze pidotimod. Pidotimod is not retained on a C_{18} column. Although pidotimod can be retained on a C_8 column, the sensitivity of the method using C_8 and C_{18} columns was much lower than the present method using HILIC. Matrix depression may be the possible reason for this difference in sensitivity. The plasma sample after protein participation may still have matrix left over in it. C_8 and C_{18} columns may not separate matrix with pidotimod satisfactorily.

The total run time was 3.5 min per sample. In contrast, the analysis times reported previously [1–5, 7] were longer than 4.5 min. The short analysis time would better meet the requirement for high sample throughput in bioanalysis.

HILIC-MS/MS operation parameters were carefully optimized for determination of pidotimod. The mass spectrometer was tuned in both positive and negative ionization modes with ESI for pidotimod analysis. The signal intensity in the positive mode was about $1.8e^6$. It was much greater than that in the negative mode, $1.3e^3$. In the precursor ion full-scan spectra, the most abundant ions were the protonated molecules $[M+H]^+$ *m/z* 245.0 and 358.2 for pidotimod and IS., respectively. The product ion scan spectra showed a high abundance of fragment ions at *m/z* 134.0 and 134.9 for pidotimod and IS, respectively (Fig. 1). Multiple reaction monitoring (MRM) using the precursor → product ion transitions of *m/z* 245.0 → *m/z* 134.0 and *m/z* 358.2 → *m/z* 134.9 was used for quantification of pidotimod and IS, respectively.

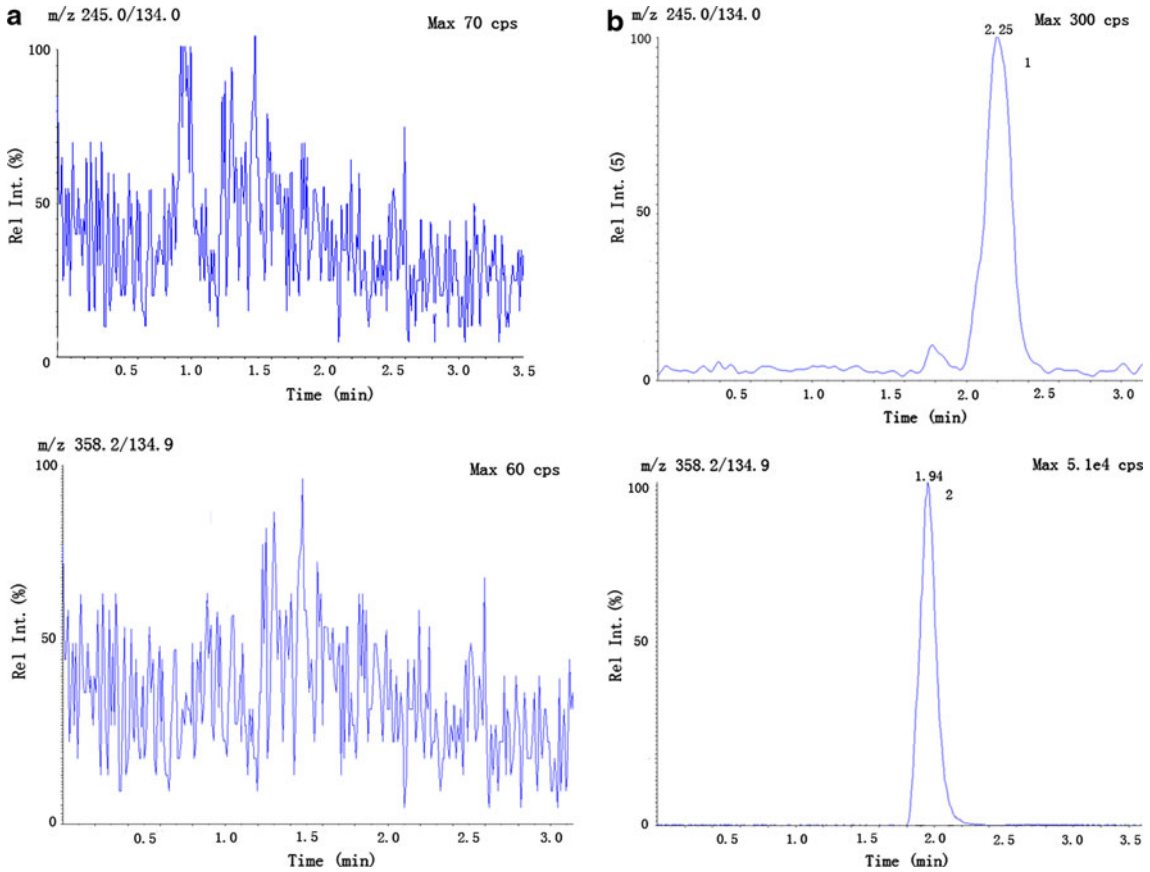

Fig. 2 **a** Representative MRM chromatograms of blank plasma samples of pidotimod (*1*) and rosiglitazone (*2*). **b** Representative MRM chromatograms of blank plasma samples spiked with pidotimod (*peak 1*) at the LLOQ of 11.2 ng mL^{-1} and rosiglitazone (*peak 2*)

Sample Preparation

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are techniques often used in the preparation of biological samples for their ability to improve the sensitivity and robustness of assays. However, pidotimod had a very high polarity; accordingly, it was better to use protein precipitation rather than LLE to prepare the plasma sample. Furthermore, protein precipitation is much simpler than LLE. Furthermore, SPE would not be cost-effective in a high-throughput analysis involving many samples. Therefore, in the present experiment, a simple protein precipitation procedure was developed to reduce sample preparation time. No further concentration procedure was needed, and the sample preparation procedure was simplified. To obtain high levels of extraction efficiency, two different protein precipitation agents, acetonitrile and methanol, were investigated. Because methanol and acetonitrile had the same effect on the extraction efficiency, methanol was chosen as the protein precipitation agent because of its lower toxicity. High extraction efficiency was achieved too when this procedure was applied to IS.

Table 1 Precision and accuracy for the determination of pidotimod in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Concentrations (ng/mL)		RSD (%)		Relative error (%)
Added	Found ± SD	Intra-day	Inter-day	
11.2	12.0 ± 0.5	4.1	1.7	7.5
28.0	25.0 ± 0.9	2.8	6.6	−10.5
1.12×10^3	$1.06 \times 10^3 \pm 61.6$	5.8	1.1	−5.7
8.96×10^3	$9.80 \times 10^3 \pm 246.2$	2.2	3.5	9.4

Finally, this simple, single-step methanol protein precipitation procedure was adopted.

Method Validation

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the

Table 2 Stability of pidotimod in plasma samples ($n = 5$)

Stability	Recovery ± RSD (%)		
	28.0 (ng/mL)	1.12×10^3 (ng/mL)	8.96×10^3 (ng/mL)
Short-term stability	87.8 ± 3.6	94.6 ± 4.7	111.6 ± 2.4
Long-term stability	86.4 ± 1.2	93.8 ± 4.8	109.5 ± 2.6
Freeze-thaw stability	90.0 ± 2.8	93.8 ± 2.0	111.6 ± 2.2
Post-preparative stability	90.7 ± 4.3	92.0 ± 4.8	107.4 ± 3.9

corresponding spiked plasma. As shown in Fig. 2a, no interference from any endogenous substance was observed at the retention time of pidotimod and the IS.

Linearity and Lower LLOQ

The standard calibration curve for pidotimod was linear over the concentration range of 11.2–1.12×10^4 ng mL^{-1} by using weighted least square linear regression analysis with a weight factor of $1/x^2$. The typical regression equation for the calibration curves for pidotimod was: $y = 4.25 \times 10^{-4}x - 1.29 \times 10^{-6}$, $r = 0.9913$.

The LLOQ for pidotimod was 11.2 ng mL^{-1} with precision and accuracy, as presented in Table 1 with RE within ±20% and RSD lower than 20%. A corresponding chromatogram is given in Fig. 2b

Precision and Accuracy

The data of intra-day and inter-day precision and accuracy for the method are listed in Table 1. The intra-day and inter-day precision for low, medium, and high concentration QC samples of pidotimod was below 6.6%, with the accuracy within the range of −10.5 to 9.4%. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples laid down by United States Food and Drug Administration (USFDA) [9] according to which the RSD at each concentration level is required to be lower than 15% and accuracy (in terms of RE) within ±15% of the actual value.

Extraction Recovery and Matrix Effect

The extract recoveries of pidotimod from human plasma were 82.3 ± 5.1, 85.6 ± 3.8, and 81.5 ± 1.6% at concentrations of 28.0, 1.12×10^3, and 8.96×10^3 ng mL^{-1}, respectively. The mean extraction recovery of rosiglitazone was 85.6 ± 1.2%.

In terms of the matrix effect, all the ratios defined as in Sect. 2 were between 85 and 115%, which means no matrix effect was observed for pidotimod in this method.

Stability

The stock solutions of pidotimod and rosiglitazone were found to be stable for 4 h at room temperature and for 30 days at 4 °C. The results from all the stability tests presented in Table 2 proved the excellent stability of pidotimod throughout all steps of the determination. The method is therefore applicable for routine analysis.

Pharmacokinetic Application

The present method was successfully applied to the pharmacokinetic study of pidotimod after oral administration to healthy volunteers. After the administration of pidotimod (800 mg), the C_{max} and T_{max} values were 4.51 ± 2.33 μg mL^{-1} and 2.5 ± 0.9 h, respectively. The concentration in plasma declined with the $t_{1/2}$ of 2.5 ± 1.1 h. The mean (±SD) $AUC_{0-\infty}$ for pidotimod in the plasma was 22.13 ± 7.5 μg h mL^{-1}. These pharmacokinetic parameters were in accordance with those reported in the literature [1, 4, 5, 7].

Conclusion

A sensitive, selective, and rapid HILIC-MS/MS method for the determination of pidotimod in human plasma is described for the first time. Compared with other published methods, this method offered superior sensitivity, with an LLOQ of 11.2 ng mL^{-1} for pidotimod, satisfactory selectivity, and a shorter run time of 3.5 min, in addition to using less plasma. According to the USFDA [9], the method can be applied for the monitoring and determination of the pharmacokinetic parameters of pidotimod.

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