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A robust LC–MS/MS method for the determination of pidotimod in different biological matrixes and its application to *in vivo* and *in vitro* pharmacokinetic studies

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ABSTRACT

Pidotimod, (*R*)-3-[(*S*)-(5-oxo-2-pyrrolidinyl) carbonyl]-thiazolidine-4-carboxylic acid, was frequently used to treat children with recurrent respiratory infections. Preclinical pharmacokinetics of pidotimod was still rarely reported to date. Herein, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated to determine pidotimod in rat plasma, tissue homogenate and Caco-2 cells. In this process, phenacetin was chosen as the internal standard due to its similarity in chromatographic and mass spectrographic characteristics with pidotimod. The plasma calibration curves were established within the concentration range of 0.01–10.00 μg/mL, and similar linear curves were built using tissue homogenate and Caco-2 cells. The calibration curves for all biological samples showed good linearity ($r > 0.99$) over the concentration ranges tested. The intra- and inter-day precision (RSD, %) values were below 15% and accuracy (RE, %) was ranged from −15% to 15% at all quality control levels. For plasma, tissue homogenate and Caco-2 cells, no obvious matrix effect was found, and the average recoveries were all above 75%. Thus, the method demonstrated excellent accuracy, precision and robustness for high throughput applications, and was then successfully applied to the studies of absorption in rat plasma, distribution in rat tissues and intracellular uptake characteristics in Caco-2 cells for pidotimod.

1. Introduction

Despite the development and usage of antibiotics and vaccines, the frequency of respiratory tract infections was still high from children to aged people because of the deficiency of their immune system, high number of circulating viruses, complex environmental risk factors, *etc.* [1–3]. Pidotimod (3-L-pyroglutamyl-L-thiazolidine-4carboxylic acid), a synthetic dipeptide molecule, was definitely confirmed to have immunomodulatory activity on both innate and adaptive responses via acting on different immunological pathways [4–8]. More importantly, many studies showed that pidotimod had a good safety profile since no serious adverse event occurred during clinical use in the past few years [2–4].

Comparing the numerous published literatures about the pharmacological studies for pidotimod, the pharmacokinetics, especially the preclinical pharmacokinetics, was still rarely reported until now. In 1994, the pharmacokinetics of pidotimod in rats, dogs and humans was studied by Coppi et al., and the biological specimen was determined based on HPLC assay [9,10]. But the low sensitivity (LLOQ higher than 100 ng/mL) was not suitable for the determination of pidotimod in rat tissues, cells, subcellular fraction or microsome. In order to enhance the sensitivity and throughput, Zhang et al. developed a HPLC–MS/MS method for the determination of pidotimod in human plasma in 2009. The method, with LLOQ at 50 ng/mL, was applied to the clinical pharmacokinetic study in healthy volunteers after oral administration of 800 mg pidotimod [11]. In 2012, Lou et al. also reported a similar LC–MS/MS method for determination of pidotimod in human plasma with LLOQ at 50 ng/mL [12]. To our knowledge, no LC–MS/MS method for the determination of pidotimod in rat plasma, tissue and cells has been reported until now. The practicability of the method reported in the previous literatures in preclinical pharmacokinetics was unknown

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since matrix effects caused by different types of biological samples were extremely diverse in LC–MS/MS analysis [13]. More importantly, the sensitivity of the analytical method should be enhanced due to low concentration levels of pidotimod in rat tissues and cells.

Caco-2 cell, an extremely powerful tool in the drug development process, was widely used as drug intestinal absorption *in vitro* model since the uptake and transport ability on Caco-2 cells was closely related to *in vivo* bioavailability and absorption [14,15]. In the present study, a robust method was developed and validated for the determination of pidotimod in rat plasma, tissues and Caco-2 cells using the AB SCIEX Triple Quad™ 5500 LC/MS/MS system. The optimized method provided a selective, sensitive, and reliable strategy for the quantitative analysis of pidotimod in actual biological samples. Moreover, the applicability of the method was validated via studying the absorption, distribution and uptake characteristics for pidotimod in rats and Caco-2 cells.

2. Experimental

2.1. Materials

Pidotimod (Lot No. 100588-201402) was purchased from National Institutes for Food and Drug Control (Beijing, China). Pidotimod oral liquid was supplied by Jiangsu Wuzhong Pharmaceutical Group Co., Ltd (Suzhou, China). The internal standard (IS) phenacetin (Item No. V900730) was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd (Shanghai, China). Acetonitrile and methanol of HPLC-grade were purchased from Merck (Merck, Germany). Formic acid (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). Deionized water was prepared by the Milli-Q system (Millipore Corporation, Billerica, MA) and was used throughout. Other reagents and solvents used for analysis were all of analytical grade.

2.2. Preparation of standards

The primary stock solutions of pidotimod and IS were prepared in 50/50 methanol/water (v/v) at the concentration of 1.0 mg/mL and 0.5 mg/mL respectively. The IS working solution was prepared by dilution with methanol to a concentration of 200 ng/mL. Pidotimod stock solution was diluted with methanol to provide working standard solutions at desired concentrations. All the solutions were stored at 4 °C and brought to room temperature before use.

For the analysis of pidotimod in rat plasma, calibration curves were constructed by spiking blank plasma at concentration of 0.01, 0.025, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/mL. Quality control (QC) samples were prepared independently in the same manner at low (0.025 μg/mL), medium 0.50 μg/mL) and high (5.00 μg/mL) levels. For the analysis of pidotimod in rat tissues, calibration curves were constructed by spiking blank liver homogenate at concentration of 0.01, 0.025, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/0.2 g tissue. Quality control (QC) samples were prepared independently in the same manner at low (0.025 μg/0.2 g tissue), medium 0.50 μg/0.2 g tissue) and high (5.00 μg/0.2 g tissue) levels. For the analysis of pidotimod in Caco-2 cells, calibration curves were constructed by spiking blank cell suspension at concentrations of 0.01, 0.025, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/mg protein. Quality control (QC) samples were prepared independently at low (0.025 μg/mg protein), medium 0.50 μg/ mg protein) and high (5.00 μg/ mg protein) levels. All the specimen were stored at −80 °C and were brought to room temperature before use.

2.3. Instrument, parameters, and conditions

2.3.1. Liquid chromatography conditions

The assay was performed on the LC-30A Shimadzu HPLC system (Kyoto, Japan) with binary pump, vacuum degasser, column oven and an autosampler system. Separation was carried out using a Sepax Bio-C18 column (150 × 2.1 mm, 5 μm, 300 A) with 0.05% formic acid (solvent A) and methanol (solvent B) as mobile phase. The gradient program (delivered at 0.3 mL/min) started at an isocratic elution of 2% of solvent B for the initial 1.0 min, then increased to 90% of solvent B from 1 to 3 min, held the composition of 90% solvent B for the next 3 min followed by column equilibration to the initial conditions over 3 min.

2.3.2. Mass spectrometry conditions

The AB SCIEX Triple Quad™ 5500 LC/MS/MS system (Framingham, MA, USA) equipped with an electrospray ionization (ESI) interface was used for quantification. In positive ion mode, the mass spectrometer was performed using MRM to monitor the mass transition pairs: m/z 245.2 → 134.2 for pidotimod, m/z 180.2 → 110.1 for phenacetin (IS). The optimized parameters were as follows: curtain gas (CUR), nebulizer gas (GS1) and turbo gas were 40, 55 and 40 psi, respectively; source temperature was maintained at 600 °C; ion spray (IS) voltage at 4500 V; the collision energy (CE) for pidotimod and IS was 20 and 30 eV, respectively. MS data acquisition was performed using Analyst TF 1.6.1 software (AB SCIEX, Framingham, MA, USA).

2.3.3. Robustness

To determine the robustness of the present developed method, effect of deliberate variations in system parameters like the content of the mobile phase additive (0.05%–0.2% formic acid), the mobile phase flow rate (0.15–0.3 mL/min), the organic component of the mobile phase (±5%), the column oven temperature (30–40 °C) was investigated systematically.

2.4. Sample preparation workflow

2.4.1. Preparation for rat plasma

The rat plasma sample (50 μL) was spiked with 10 μL of internal standard solution and mixed for 10 s, and 250 μL of acetonitrile was then added to precipitate protein. After mixing for 30 s, the mixture was centrifuged at 45,000*g* for 10 min, and 150 μL of supernatant was transferred to an autosampler vial. Finally, an aliquot of 10 μL sample was injected into the LC–MS/MS system for analysis.

2.4.2. Preparation for rat tissues

A total of 0.2 g tissues (heart, liver, spleen, kidney, thymus, pancreas, thyroid, brain, stomach, and intestines) were homogenized in 500 μL deionized water with a tissue homogenizer (IKA ULTRA-TURRAX T25 model, Germany) in an ice bath. Subsequently, the homogenates (50 μL) were spiked with 10 μL of internal standard solutions and mixed, and 250 μL of acetonitrile was then added to precipitate protein. After mixing on vortex mixer for 30 s and centrifugation at 45,000*g* for 10 min, 400 μL of supernatant was transferred to a fresh Eppendorf tube and centrifuged again at 45,000 g for 10 min. Finally, a 10 μL aliquot was injected into the LC–MS/MS system for analysis.

2.4.3. Preparation for Caco-2 cell

After incubation pidotimod (100, 200 and 500 μg/mL) for 2 h at 37 °C with Caco-2 cell, the cellular uptake was stopped by rapidly aspirating the drug solutions, and the cells were washed for three times with ice-cold uptake buffer for three times. Cellular protein concentrations were determined using a protein assay kit