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Simultaneous determination of roflumilast and its metabolite in human plasma by LC–MS/MS: Application for a pharmacokinetic study

Xinge Cui [a,1], Jie Huang [b,1], Xin Zheng [a], Ji Jiang [a], Yun Kuang [b], Pei Hu [a,*]

[a] Clinical Pharmacology Research Center, Peking Union Medical College Hospital, China
[b] Center of Clinical Pharmacology, Third Xiangya Hospital, Central South University, China

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ABSTRACT

Roflumilast had shown good efficacy and safety in Caucasian COPD patients after oral administration of 0.5 mg. The main active metabolite of it is roflumilast *N*-oxide. A reliable liquid chromatography–tandem mass spectrometry (LC–MS/MS) quantitation method was developed for the simultaneous determination of them in human plasma with rather low limits of quantitation for roflumilast (0.02 ng/mL) and roflumilast *N*-oxide (0.04 ng/mL). Human plasma samples were prepared by solid phase extraction (SPE), which ensured high recovery and slight matrix effect for the both analytes. This method showed good linearity, accuracy, precision and stability in the range of 0.02–10 ng/mL and 0.04–50 ng/mL for roflumilast and roflumilast *N*-oxide respectively. The developed method was successfully applied for the pharmacokinetic research in Chinese healthy volunteers after oral administration of 0.25 mg, 0.375 mg and 0.5 mg of roflumilast tablet.

1. Introduction

Roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-*N*-[3,5-dichloropyridyl-4]-benzamide), which is an oral inhibitor of phosphodiesterase 4, is a kind of non-steroidal anti-inflammatory drug. It is used for the treatment of systemic and pulmonary inflammation associated with chronic obstructive pulmonary disease (COPD). It has been proved that roflumilast *N*-oxide is the main metabolite of roflumilast in human plasma, which contributes to the efficacy of roflumilast largely in vivo [1]. The inhibition of phosphodiesterase 4 by roflumilast and roflumilast *N*-oxide leads to enhanced intracellular level of cyclic adenosine monophosphate, which could active protein kinase A and result in the reduction of cellular inflammatory activity [2]. It has been proved in several randomized clinical trials that roflumilast could improve prebronchodilator forced expiratory volume in 1 s, postbronchodilator forced expiratory volume in 1 s and decrease the rate of exacerbations in Caucasian COPD patients [3–5] without potent adverse effect after oral administration of 0.5 mg of roflumilast.

Due to the good efficacy and safety of roflumilast in Caucasian COPD patients, it is in urgent need of the introduction of roflumilast to Chinese patients. Roflumilast is mainly metabolized by CYP3A4 and CYP1A2. The frequency and activity of CYP1A2 show a significant difference between Caucasian and Chinese [6–8], which might result in the difference in the exposure of rolumilast and roflumilast *N*-oxide between Caucasian COPD patients and Chinese COPD patients. Moreover, it has been proved that the adverse effect profile of roflumilast is different in Asian COPD patients from that in Caucasian COPD patients [4,5,9], which might be related to the difference in exposure.

Several quantitation methods of roflumilast and roflumilast *N*-oxide have been developed. Knebel et al. [10] developed a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for roflumilast and roflumilast *N*-oxide with liquid–liquid extraction (LLE) being the sampled preparation method. The lower limits of quantitation (LLOQs) were 0.1 ng/mL for both roflumilast and roflumilast *N*-oxide. Another quantitation method about roflumilast and roflumilast *N*-oxide was developed by Thappali et al. [11] with the LLOQs being 0.101 ng/mL and 0.111 ng/mL respectively. In this method, the analytes were extracted from plasma by *tert*-Butyl methyl ether and then detected by LC–MS/MS. Taking the exposure differences between Chinese and Caucasian and the microgram level administration of roflumilast into consideration, plasma concentrations of roflumilast and roflumilast *N*-oxide might be rather low in Chinese population and the existing quantitation methods

* Corresponding author at: No. 41, Damucang Hutong, Xicheng District, Beijing, 100032, China.
E-mail address: pei.hu.pumch@gmail.com (P. Hu).
[1] Xinge Cui and Jie Huang contributed equally to this work.

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might not meet the requirement of the analysis of them in Chinese. So a new quantitation method with much lower LLOQs for roflumilast and roflumilast *N*-oxide should be developed for the investigation of pharmacokinetics of roflumilast in Chinese. In this assay, a quantitation method was developed with lower LLOQs for both roflumilast and roflumilast *N*-oxide than the published quantitative method [10,11], in order to get more detailed pharmacokinetic information of both of them in Chinese.

2. Experiment

2.1. Standards, reagents & materials

Standards of roflumilast and roflumilast *N*-oxide were provided by Baili Pharmaceutical Limited liability company (Sichuan, China), who was the sponsor of the pharmacokinetic research of roflumilast tablet in Chines healthy volunteers. Standards of [D_4]-roflumilast and [D_4]-roflumilast *N*-oxide, which were purchased from TLC Pharmaceutical Standards Limited company (Ontario, Canada), were used as internal standards for roflumilast and roflumilast *N*-oxide, respectively. Their chemical structures are shown in Fig. 1. All solvents (acetonitrile-Fisher Scientific, NJ, USA; methanol-Fisher Scientific, NJ, USA) were HPLC grade. Formic acid (Sigma-Aldrich, MO, USA) and ammonium acetate (Sinopharm Chemical Reagent Ltd, Shanghai, China) used in this study were analytical pure. Water used in this study was purified by a Milli-Q® system (Millipore, MA, USA). Blank plasma, which was collected from healthy volunteers with heparin lithium as anticoagulant, was stored in freezer at −30 °C ever since collection.

2.2. Preparations of stock solutions, calibration standards and quality control (QC) samples

Stock solutions of roflumilast, roflumilast *N*-oxide, [D_4]-roflumilast, [D_4]-roflumilast *N*-oxide were prepared in methanol respectively, and the concentrations were all 1 mg/mL. For the analytes of this study, namely roflumilast and roflumilast *N*-oxide, two sets of stock solutions were prepared. One set was used to prepare the working solutions of calibration standards, and the other set was used to prepare the working solutions of QC samples. All stock solutions were stored at −80 °C.

Working solutions of analytes were prepared with mixed blank plasma, which was prepared by mixing blank plasma from six different healthy volunteers with the same volume. All working solutions were fresh and prepared at desired concentrations for the preparation of calibration standards and QC samples. Stock solutions of [D_4]-roflumilast and [D_4]-roflumilast *N*-oxide were diluted together with methanol-water (20:80, v/v) for the preparation of internal standard solution (IS), of which the concentrations were both 10 ng/mL for [D_4]-roflumilast and [D_4]-roflumilast *N*-oxide.

Calibration standards, which were prepared by spiking an appropriate amount of working solutions of analytes into mixed blank plasma, were subpackaged into appropriate volumes and stored at −30 °C during the valid period. Eight calibration standards of analytes were prepared at 0.02, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, 10 ng/mL for roflumilast and 0.04, 0.1, 0.2, 1.0, 5.0, 10, 25, 50 ng/mL for roflumilast *N*-oxide. Preparation methods and storage conditions of QC samples were same to those of calibration standards. The concentrations of lower limit of quantitation (LLOQ) sample, lower quality control (LQC) sample, middle quality control (MQC) sample, higher quality control (HQC) sample and dilution quality control (DQC) sample were 0.02, 0.06, 0.8, 8, 20 ng/mL for roflumilast, and 0.04, 0.12, 2, 40, 100 ng/mL for roflumilast *N*-oxide.

2.3. Sample preparation

Plasma samples were prepared by solid phase extraction (SPE). Aliquot of 200 μL of blank plasma, calibration standard, QC samples or human plasma was mixed with 100 μL of IS and 200 μL of water. The mixture was totally mixed for 30 s and instantly centrifuged for 10 s at 17,000*g*. In order to extract the analytes and internal standards, the mixture was loaded to Waters Oasis® HLB 96-well plate (10 mg, 30 μm) (Waters, MA, USA) subsequently, which had been previously conditioned with 1 mL of methanol and 1 mL of purified water. After extraction, the cartridges were washed by 1 mL of methanol-water (20:80, v/v) and reduced pressure was applied until the cartridges were dry. Then, analytes and internal standards were eluted with 0.4 mL of methanol twice, and reduced pressure was applied until the cartridges were dry. Finally, eluents were evaporated to dryness at room temperature and reconstituted with 100 μL of acetonitrile-water (50:50, v/v).

2.4. Analysis system

Liquid chromatographic (LC) analysis was performed with an ACQUITY UPLC system (Waters, MA, USA). Mobile phase A (MPA) was consisted of formic acid-10 mM ammonium acetate (1:1000, v/v) and mobile phase B (MPB) was acetonitrile. Separation was achieved by an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) from Waters (MA, USA) under a gradient elution of 50% of MPB (0–0.5 min), from 50% to 60% of MPB (0.5–1.5 min), 60% of MPB (1.5–2.5 min), from 60% to 50% (2.5–2.51 min) and 50% of MPB (2.51–3.0 min). The column temperature was set to be 40 °C and the autosampler temperature was set to be 8 °C.

Mass spectrometry (MS) detection was achieved by API 5500 Q-trap® (MDS-AB SCIEX, Concord, Canada) equipped with ESI ion source. Mass detection was operated in positive mode with mass spectrometry parameters as follows: temperature, 550 °C; gas1, 60; gas2, 60; curtain gas, nitrogen, 30; collision gas, 7. Multiple reaction monitoring mode was used for quantitation. For roflumilast, declustering potential (DP) was set to be 160 V and the entrance potential (EP) was set to be 10 V. For roflumilast *N*-oxide, DP was set to be 136 V and EP was set to be 10 V. For [D_4]-roflumilast, DP and EP were set to be 146 V and 10 V, respectively. For [D_4]-roflumilast *N*-oxide, DP and EP were set to be 161 V and 10 V, respectively. Transition m/z 403.1 → 187.1 and transition m/z 403.1 → 241.1 were monitored for roflumilast, of which the former was used for quantitation and the latter was used for confirmation. The collision energy (CE) and the collision cell exit potential (CXP) for the former transition of roflumilast were 35 eV and 10 V respectively, which were 25 eV and 12 V for the latter transition. Transition m/z 419.1 → 187.1 and transition m/z 419.1 → 241.1 were monitored for roflumilast *N*-oxide, of which the former was used for quantitation and the latter was used for confirmation. The CE and the CXP for the former transition of roflumilast *N*-oxide were 37 eV and 10 V respectively, which were 25 eV and 12 V for the latter transition. In addition, transition m/z 407.1 → 245.2 was monitored for [D_4]-roflumilast with CE and CXP being 25 eV and 12 V respectively. Transition m/z 423.1 → 245.2 were monitored for [D_4]-roflumilast *N*-oxide with CE and CXP being 27 eV and 12 V respectively. The dwell time of all the above transitions were 100 ms.

2.5. Data analysis

All data used for quantitation were acquired and analyzed by Analyst software (version 1.5.1) of AB SCIEX (Concord, Canada). A linear regression was applied with concentration being the explanatory variable and peak area ratio of analyte to the corresponding internal standard being the explained variable, of which the weighting factor was $1/x^2$. The bias of the calibration standards