Journal of Pharmaceutical and Biomedical Analysis 94 (2014) 125–131

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Simultaneous quantification of ruxolitinib and nilotinib in rat plasma by LC–MS/MS: Application to a pharmacokinetic study

Sridhar Veeraraghavan[a,c,*], Satheeshmanikandan Thappali[a], Srikant Viswanadha[a], Sandhyarani Chennupati[a], Santhoshkumar Nalla[a], Manikantakumar Golla[a], Swaroopkumar Vakkalanka[a], Manivannan Rangasamy[b]

[a] *Incozen Therapeutics Private Limited, 450, Alexandira Knowledge Park, Turkapplly, Hyderabad 500078, Andhra Pradesh, India*
[b] *Department of Pharmaceutics, Annai JKK Sampoorani Ammal College of Pharmacy, Komarapalayam, Namakkal 638183, Tamilnadu, India*
[c] *CRD, PRIST University, Vallam, Thanjavur 613403, Tamilnadu, India*

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 22 January 2014
Accepted 25 January 2014
Available online 3 February 2014

Keywords:
Ruxolitinib
Nilotinib
Plasma
LC–MS/MS

ABSTRACT

Efficacy assessments using a combination of ruxolitinib and nilotinib necessitate the development of a high precision analytical method for determination of both drugs in plasma. A high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of ruxolitinib and nilotinib in rat plasma. Extraction of ruxolitinib, nilotinib and dasatinib (internal standard; IS) from 50 μl rat plasma was carried out by protein precipitation with methanol. Chromatographic separation of analytes was performed on YMC pack ODS AM (150 mm × 4.6 mm, 5 μm) column under gradient conditions with acetonitrile:2.0 mM ammonium acetate buffer as the mobile phase at a flow rate of 1 ml/min. Precursor ion and product ion transition for both analytes and IS were monitored on a triple quadrupole mass spectrometer, operated in the selective reaction monitoring with positive ionization mode. Method was validated over a concentration range of 0.16–247 ng/ml for ruxolitinib and 0.86–219 ng/ml for nilotinib. Mean extraction recovery for ruxolitinib, nilotinib, and IS of 99.6%, 97.6% and 90.3% were consistent across low, medium, and high QC levels. Precision and accuracy at low, medium and high quality control levels were less than 15% across analytes. Bench top, wet, freeze-thaw and long term stability were evaluated for both analytes. The analytical method was applied to support a pharmacokinetic study of simultaneous estimation of ruxolitinib and nilotinib in Wistar rat. Assay reproducibility was demonstrated by re-analysis of 18 incurred samples.

1. Introduction

Chronic myeloid leukaemia (CML) is a slow, progressing, myeloproliferative disease of the bone marrow that results in overproduction of abnormal white blood cell (WBC) [1]. Historically, CML has been linked to gene rearrangement of ABL-kinase known as the “Philadelphia Chromosome” and is treated with broad-spectrum tyrosine kinase inhibitors including nilotinib [2,3]. While nilotinib demonstrated significant success in attenuating disease progression in CML, resistance to the drug has been reported in the recent past [4]. Resistance to nilotinib has been attributed to the activation of the JAK/STAT signalling pathway [5]. Combination of a JAK inhibitor with nilotinib could therefore help overcome the resistance in a sub-sect of CML patients.

Janus kinases are non-receptor tyrosine kinases that mediate the downstream effects of several cytokines and growth factors. Aberrations of the janus kinase (JAK) signalling pathway are associated with majority of the myelofibrotic cases with over 50% of primary myelofibrosis patients presenting a JAK2V617F gain-of-function mutation [6]. Such mutations result in constitutive activation of the JAK-signal transducer and activator of transcription (JAK-STAT) pathway [6] that in turn regulates transcription of several genes involved in the immune response, cell proliferation, and apoptosis [7,8]. The JAK-STAT pathway could also be triggered due to excessive production of pro-inflammatory cytokines thereby leading to a vicious cycle wherein the receptor remains activated [9]. Besides JAK2, studies have also implicated the involvement of JAK1 in the progression of myelofibrosis [9] thereby implicating these two proteins as potential druggable targets. With the discovery of JAK2 mutations in myelofibrosis, focus shifted to targeted JAK inhibitors

---

* Corresponding author at: Incozen Therapeutics Private Limited, 450, Alexandria Knowledge Park, Turkapplly Hyderabad 500078, Andhra Pradesh, India.
Tel.: +91 40 2348 0336; fax: +91 4023480337.
E-mail address: sridharveeraraghavan@gmail.com (S. Veeraraghavan).

0731-7085/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2014.01.040

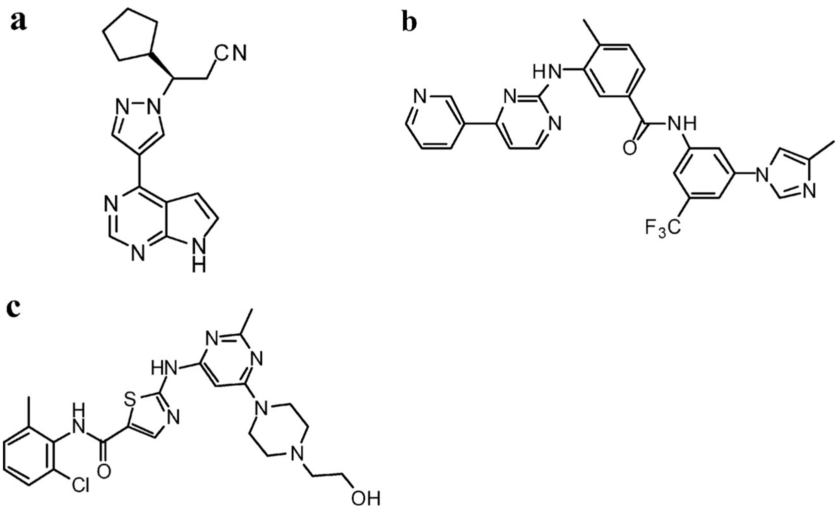

Fig. 1. Chemical structure of (a) ruxolitinib, (b) nilotinib and (c) dasatinib (internal standard).

for effective disease control. Ruxolitinib (formerly INCB018424) is first-in-class, potent, and selective JAK inhibitor that has been approved by the FDA for the treatment of myelofibrosis. A Phase 1/2 clinical trial evaluating the effect of the combination of ruxolitinib and nilotinib in CML is currently ongoing (NCT01702064).

Very few LC–MS/MS based methods are reported for the quantification of ruxolitinib in plasma [10,11]. Methods for the determination of nilotinib in biological fluids by HPLC-UV or LC–MS/MS have been reported [12–23]. However, reports describing a LC–MS/MS-based method for simultaneous determination of ruxolitinib and nilotinib in plasma are not available. Simultaneous determination of ruxolitinib and nilotinib in plasma would help establishing a pharmacokinetic and pharmacodynamic study in animal models that require administration of both drugs to achieve maximal efficacy prior to human studies. In the current article, we describe a highly sensitive, selective, and rapid LC–MS/MS method that was developed and fully validated for simultaneous estimation of ruxolitinib and nilotinib in rat plasma. This method offers a small turnaround time for analysis and utilizes only 50 μl rat plasma for sample processing using simple protein precipitation extraction. Translation of this methodology to pharmacokinetic studies is also demonstrated by re-analysis of incurred sample.

2. Experimental

2.1. Chemicals and reagents

Ruxolitinib, nilotinib, and dasatinib (Fig. 1) were obtained from Selleckchem, Houston, TX. Ammonium acetate was obtained from Sigma–Aldrich Germany. Methanol and acetonitrile (HPLC gradient grade) were procured from RCI Lab Scan, Thailand. Ultra pure water of 18 MΩ/cm was obtained from Milli-Q purification system, Millipore, MA, USA.

2.2. Liquid chromatographic and mass spectrometric conditions

Reverse phase chromatographic analysis of analytes was achieved on a Shimadzu SIL-20 AC HT system (Shimadzu Corporation, Japan). Separation of analytes and IS was performed on YMC Pack ODS AM (150 mm × 4.6 mm, 5 μm) analytical column (YMC®-PACK, Japan), maintained at 40 °C in a column oven (CTO-10AS$_{VP}$). Five microlitres of each sample were loaded on the column, separated, and eluted using a gradient mobile phase consisting of acetonitrile (A):2 mM ammonium acetate buffer (B); (minutes, % mobile phase A): (0, 25), (2.0, 90), (5.0, 90), (5.2, 25), (7.5, 25). For gradient elution, the flow rate of the mobile phase was kept at 1.0 ml/min with 70% flow split after post column elution. Flow was directed to the ion spray interface. Autosampler (SIL20AC$_{HT}$) temperature was maintained at 10 °C. Mass spectrometric detection of analytes and IS was carried out on a triple quadrupole mass spectrometer (Thermo Scientific – Finnigan TSQ Quantum Ultra, San Jose, CA, USA), equipped with a heated electrospray ionization and operated in a positive ionization mode. Optimized mass parameters and SRM transitions for analytes and IS are given in Table 1. Selective reaction monitoring (SRM) mode was used for data acquisition. Peak integration and calibration were carried out using LC Quan 2.5.2 software (Thermo- Scientific).

2.3. Calibration standard and quality control samples

Stock solutions (1.0 mg/ml) of ruxolitinib, nilotinib, or dasatinib were prepared by dissolving accurately weighed amounts in methanol. Calibration standards (CSs) and quality control (QC) samples were made by spiking blank plasma with appropriate volumes of working solutions. Final calibration standard concentrations for ruxolitinib/nilotinib were 0.16/0.86, 0.41/1.71, 1.02/3.43, 2.54/6.85, 6.35/13.71, 15.87/27.41, 39.68/54.83, 99.19/109.65, and 247.97/219.30 ng/ml respectively. The QC samples were prepared at four concentration levels: 190.75/180.60 ng/ml (HQC, high quality control), 95.37/90.30 ng/ml (MQC, medium quality control), 0.49/2.44 ng/ml (LQC, low quality control) and 0.16/0.86 ng/ml (LLOQ QC, lower limit of quantification quality control), for the ruxolitinib/nilotinib combination. Dasatinib (IS) stock solution was diluted with methanol to achieve a final concentration of 250 ng/ml. Standard stock and working solutions were stored at 2–8 °C until further use.

2.4. Extraction procedure

Analytes were extracted from rat plasma by protein precipitation. Briefly, 300 μl of precipitating solution (IS 250 ng/ml) was added to an aliquot of 50 μl plasma and mixed for 3 min (IKA vortex, Genius 3). Mixture was centrifuged at 14,000 rpm at 10 °C for 5 min. Supernatant (5 μl) was injected in the chromatographic system.

2.5. Validation procedures

System suitability was determined by injecting six consecutive samples of aqueous standard mixture of analytes and IS at the start