Journal of Chromatography B, 989 (2015) 1–10

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Micropreparative isolation and NMR structure elucidation of metabolites of the drug candidate 1-isopropyl-4-(4-isopropylphenyl)-6-(prop-2-yn-1-yloxy) quinazolin-2(1H)-one from rat bile and urine

Joachim Blanz[a,*], Thierry Délémonté[a], David Pearson[a], Alexandre Luneau[b], Michael Ritzau[b], Werner Gertsch[a], Philippe Ramstein[a], Jérôme Dayer[a], Sandrine Desrayaud[c], Elisabeth Braun[c], Reiner Aichholz[a]

[a] Analytical Sciences & Imaging, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland
[b] Global Discovery Chemistry, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland
[c] Metabolism & Pharmacokinetics, Novartis Institutes for Biomedical Research, CH-4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 6 October 2014
Accepted 27 February 2015
Available online 10 March 2015

Keywords:
Biotransformation
Drug metabolism
Metabolite identification
Micropreparative isolation
HPLC-MS-SPE-NMR

ABSTRACT

LC-MS based drug metabolism studies are effective in the optimization stage of drug discovery for rapid partial structure identification of metabolites. However, these studies usually do not provide unambiguous structural characterization of all metabolites, due to the limitations of MS-based structure identification. LC-MS-SPE-NMR is a technique that allows complete structure identification, but is difficult to apply to complex *in vivo* samples (such as bile collected during *in vivo* drug metabolism studies) due to the presence, at high concentrations, of interfering endogenous components, and potentially also dosage excipient components (e.g. polyethylene glycols). Here, we describe the isolation and structure characterization of seven metabolites of the drug development candidate 1-isopropyl-4-(4-isopropylphenyl)-6-(prop-2-yn-1-yloxy) quinazolin-2(1H)-one from a routine metabolism study in a bile-duct cannulated rat by LC-MS-SPE. The metabolites were isolated from bile and urine by repeated automatic trapping of the chromatographic peak of each metabolite on separate Oasis HLB SPE columns. The micropreparative HPLC/MS was performed on an XBridge BEH130 C18 HPLC column using aqueous formic acid/acetonitrile/methanol as mobile phase for the gradient elution. Mass spectrometric detection was performed on a LTQ XL linear ion trap mass spectrometer using electrospray ionization. Desorption of each metabolite was performed after the separation sequence. NMR spectra (^{1}H, ^{13}C, 2D ROESY, HSQC and HMBC were measured on a Bruker AVANCE III spectrometer (600 MHz proton frequency) equipped with a 1.7 mm ^{1}H{^{13}C,^{15}N} Bruker Biospin's TCI MicroCryoProbe™.

1. Introduction

Drug metabolism studies are used in early phase drug discovery (i) to identify metabolic soft spots associated with high clearance, (ii) to detect potentially toxic/reactive metabolites, and iii) for the early investigation of species differences in metabolism [1,2]. These early phase studies generally involve LC-MS analysis of samples either from *in vivo* studies or from *in vitro* incubations (e.g. with liver microsomes or hepatocytes). LC-MS analysis allows rapid identification and partial structural characterization of metabolites, with high sensitivity for most drug compounds and metabolites. However, a major drawback of MS analysis is that the quality of structural characterization depends on the collision-induced dissociation of the protonated molecular ions of the compound of interest, and therefore in most cases cannot deliver an exact metabolite structure.

NMR is a complementary analytical approach that allows more detailed structural characterization of metabolites. However, NMR is less sensitive than MS, and the NMR signal is strongly affected by the presence of impurities in a sample. As such, good separation of analyte from impurities is required to perform NMR structural identification. For this purpose, LC-NMR analyses for the characterization of metabolites have been used extensively [3–6]. However, online LC-NMR coupling suffers from a number of problems, in

* Corresponding author. Tel.: +41 61 3240445; fax: +41 61 3240342.
E-mail address: Joachim.Blanz@novartis.com (J. Blanz).

http://dx.doi.org/10.1016/j.jchromb.2015.02.044
1570-0232/

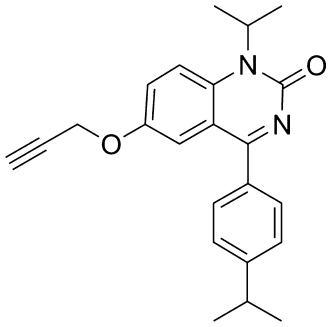

Fig. 1. Chemical structure of **1**.

particular that the concentration of compounds after LC separation and transfer to a NMR flow cell is often too low for NMR analysis. LC-MS-SPE-NMR is a recently reported NMR method that improves on this situation. The LC-MS-SPE-NMR approach involves the analysis of sample by LC-MS with simultaneous SPE isolation of sample components of interest. Subsequently, the isolated compounds are transferred to an online or offline NMR for structural characterization. This method allows compounds of interest to be pre-concentrated before NMR analysis, resulting in improved overall sensitivity. Decoupling of the chromatographic separation from the NMR detector also allows replacement of the conventional room temperature probe by cryogenically cooled probes with higher NMR sensitivity [7].

The LC-MS-SPE-NMR approach has been successfully applied to the structural characterization of metabolites in drug metabolism studies [8–11], as well as various other compounds including plant [12] and human [13] endogenous metabolites, chemical impurities [14] and packaging extractables [15]. The reported use of this approach for drug metabolism is largely limited to *in vitro* studies, and there are few reports of LC-MS-SPE-NMR characterization of metabolites from *in vivo* samples [8,11]. It is likely that this approach is not often used for *in vivo* metabolism studies due to difficulties of separating compounds of interest from highly concentrated endogenous components and other impurities. Here, we report the isolation of seven metabolites from bile and urine obtained from a routine *in vivo* metabolism study in a bile-duct cannulated rat with a non-radioactively labelled drug candidate **1** ((1-isopropyl-4-(4-isopropylphenyl)-6-(prop-2-yn-1-yloxy) quinazolin-2(1H)-one), Fig. 1). Compound **1** is a calcium-sensing receptor (CaSR) antagonist investigated for the treatment of osteoporosis. To the best of the authors' knowledge, this represents the first report of a full LC-MS-SPE-NMR characterization of drug metabolites in a routine *in vivo* metabolism study.

2. Materials and methods

2.1. Chemicals and reagents

CHROMASOLV® Plus HPLC grade water (Sigma, Buchs, Switzerland) was further purified by distillation using a Water Still Distinction D4000 (GMB Glasmechanik, Therwil, Switzerland). CHROMASOLV® HPLC grade acetonitrile (MeCN) and methanol (MeOH), phosphate buffer (PBS, 1.0 M pH 7.4), trifluoroacetic acid (TFA), N-methyl-2-pyrrolidone (NMP) and polyethylene glycol-200 (PEG200) were purchased from Sigma (Buchs, Switzerland). Optima LC/MS grade methanol and acetonitrile were purchased from Fisher Scientific (Waltham, Massachusetts USA). CHROMASOLV ® NMR grade acetonitrile (used for metabolite desorption), ammonium formate and formic acid were purchased from Fluka (Buchs, Switzerland). Nitrogen (purity 99.5%) and Argon (purity 99.995%) were purchased from Carbagas (Lausanne, Switzerland). Perdeuterated dimethylsulfoxide (DMSO-d6, 100 atom% D) was purchased from Armar Chemicals (Döttingen, Switzerland). Oasis HLB columns and SPE cartridges were purchased from Waters (Milford, Massachusetts, USA). Compound **1** (1-isopropyl-4-(4-isopropylphenyl)-6-(prop-2-yn-1-yloxy)quinazolin-2(1H)-one) was synthesized by Rene Beerli (Novartis Institutes for Biomedical Research, Basel, Switzerland).

2.2. Bile and urine samples

The *in vivo* experiment was performed according to the regulations effective in the Canton Basel-City, Switzerland, specifically according to experimental license No. 2241. One Sprague Dawley male rat (Crl:CD(SD)), obtained from Charles River Laboratories (Iffa-Credo, France) weighing 320 g was used for this study. Before the surgery, the rat was kept under standard conditions (optimal health conditions [OHC], 22 °C in a special, acclimatized animal room with 12 h dark–light cycles, light from 06:00 to 18:00) with free access to tap water and pelleted rodent chow. The day before drug administration, the animal was anaesthetized and then, under aseptic conditions, three catheters were successively implanted and fixed into (i) its femoral artery for blood collection, (ii) its femoral vein for drug administration and (iii) in its bile duct for continuous bile collection. After the surgery (i.e. after cannulation of the bile duct and of the blood vessels) and for the experiment, the animal was individually placed in a metabolic cage and connected to the freely moving Harvard swivel system. In order to allow good ionic balance during the experimental period (i.e. between surgery and sacrifice), drink water was replaced by Ringer solution (glucose 5%, NaCl 0.9% and KCl 0.5%). The rat had always free access to food. Due to the continuous bile flow (~1 mL/h), the recovery period after the surgery was about 24 h and the duration of the study (i.e. after drug injection) was 8 h post-dose. Compound **1** (3 mg/kg, 0.5 mL/kg a solution in NMP/PEG, 30:70, v/v) was administered intravenously *via* the catheter implanted into the femoral vein. Bile and urine were collected quantitatively (i.e. excreta volumes were recorded) at pre-defined time intervals (0–2 h, 2–4 h, 4–6 h, 6–8 h) without disturbing the animal. Blood was also withdrawn *via* the catheter implanted into the femoral artery at 1, 4 and 8 h post-dose. All samples were collected on ice, and then frozen as soon as possible at −80 °C until analysis.

2.3. Analytical capillary high-performance liquid chromatography–mass spectrometry (HPLC/MS)

Capillary HPLC was performed on a system consisting of a Chorus-220 HPLC pump (CTC Analytics, Zwingen, Switzerland), a HotDog-5090 column oven (Prolab, Reinach, Switzerland) and a HTS-PAL autosampler with cooled sample stacks (CTC Analytics, Zwingen, Switzerland). Separations were performed on a Reprosil Basic HD C18 HPLC column (150 mm × 0.3 mm i.d., 3.0 μm particle size) from Maisch (Ammerbuch-Entringen, Germany). Separations were performed at 40 °C. The flow rate was 4.5 μL/min and the injection volume was 1 μL per separation. The solvent system used consisted of aqueous ammonium formate (10 mM, with 0.02% TFA, pH 4)/MeCN (95/5, v/v) as solvent A and aqueous ammonium formate (10 mM, with 0.02% TFA, pH 4)/MeCN/MeOH (5/90/5, v/v/v) as solvent B. The metabolites were separated using a linear solvent gradient: 0 min (5% B), 2 min (5% B), 27 min (95% B), 32 min (95% B, 6.5 μL/min). Re-equilibration of the column was performed at 5% B for 5 min. Prior analysis, the 10 μL of sample was diluted 1/100 (v/v) with water/MeCN (90/10, v/v). Samples were kept in the autosampler at 10 °C.

A LTQ XL Orbitrap (Linear Quadrupole 2D Ion Trap/Orbitrap, Thermo Scientific, CA, USA) mass spectrometer equipped with a captive spray ionization (CSI) source (Microchrom Bioresources,