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Sampling only ten microliters of whole blood for the quantification of poorly soluble drugs: Itraconazole as case study

Justine Thiry[a], Brigitte Evrard[a], Gwenaël Nys[b], Marianne Fillet[b], Miranda G.M. Kok[b,*]

[a] Laboratory of Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, Centre for Interdisciplinary Research on Medicines (CIRM), University of Liège, Quartier Hopital, Avenue Hippocrate 15, 4000, Liège, Belgium
[b] Laboratory for the Analysis of Medicines, CIRM, University of Liège, Quartier Hopital, Avenue Hippocrate 15, 4000, Liège, Belgium

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ABSTRACT

Nowadays in animal studies, it is important to comply with the so-called Three Rs rule by replacing or reducing the number of tested animals. Volumetric absorptive microsampling (VAMS) can be used to collect small quantities (10 or 20 μL) of whole blood, thereby limiting the amount of animals needed. In this study, a quantitative method was developed and subsequently validated for the poorly soluble drug itraconazole (ITZ) using VAMS and ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS). A proof of concept study showed that the optimized method is applicable to test the bioavailability of drug formulations containing ITZ. Using VAMS, smaller blood volumes can be taken per sampling point (10–20 μL instead of the conventional 0.2-0.5 mL) avoiding the sacrifice of animals. Moreover, the same rats can be used to compare different drug formulations which strengthens the validity of the results. In long-term bioavailability studies, it is necessary to guarantee the stability of the tested drugs supported on VAMS devices. In this study, we show that ITZ was only stable for 24 h after collection with VAMS, but for at least two weeks by the storage of extracted samples at −80 °C.

1. Introduction

'The Three Rs rule' – replacement, reduction and refinement – have been introduced by Russell and Burch in 1959 [1]. The Three Rs is a set of guiding principles for more ethical use of animals used in scientific studies. Nowadays, complying with this rule is of utmost importance and efforts have to be made to replace or reduce the number of animals in testing. In addition, pain and distress during studies have to be minimized to enhance the animal welfare.

A lot of animals are generally required in bioavailability studies in which multiple drug formulations are compared. This is because significant volume blood samples have to be drawn at different time points in order to obtain reliable pharmacokinetic profiles. Therefore, at least one animal per formulation is needed resulting in a large number of animals. Thus, adjustments are indicated in order to meet the Three Rs rule. In the framework of this rule, volumetric absorptive microsampling (VAMS) has been developed to collect small quantities of whole blood [2]. A VAMS device consists of a porous hydrophilic tip attached to a sample handler. This tip has been designed to collect a fixed volume (10 or 20 μL) through wicking. The collected blood needs to be dried for two hours after which it can be shipped and/or stored before analysis of the blood components of interest. Besides the compliance to the reduction and refinement concept, another advantage of VAMS is the collection of a constant blood volume regardless of the hematocrit and the homogeneity of samples. For instance, with VAMS a variation of less than 4% was observed for caffeine across a wide range of hematocrit levels (20–70%), compared to a variation of 30% using another sampling technique with dried whole blood, namely the dried blood spot (DBS) technique. Moreover, issues of non-homogeneity experienced by DBS samples (using only a part of the dried spot for analysis) are negated with the VAMS technique, since the whole sampling tip can be used for the extraction of analytes [2–4].

VAMS has shown great potential for the analysis of whole blood samples [2–7]. Mostly, blood samples spiked with compounds belonging to class I of the Biopharmaceutics Classification System (BCS) have been analysed with VAMS. These compounds are char-

Abbreviations: ACN, acetonitrile; BCS, biopharmaceutics classification system; DBS, dried blood spot; FA, formic acid; FDA, food and drug administration; HPLC, high performance liquid chromatography; IPA, isopropanol; ITZ, itraconazole; MeOH, methanol; MRM, multiple reaction monitoring; MS, mass spectrometry; OH-ITZ, hydroxy-itraconazole; TFA, trifluoroacetic acid; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet; VAMS, volumetric absorptive microsampling.

* Corresponding author.
E-mail address: miranda.kok@ulg.ac.be (M.G.M. Kok).

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acterized by a good water solubility and membrane permeability [8], thereby facilitating a good recovery of analytes from the VAMS devices. The analysis of poorly soluble drug molecules using VAMS might be a bigger challenge. Since more than 70% of active pharmaceutical ingredients in the pipeline of the pharmaceutical industry are considered as poorly soluble [9], the development of a quantitative method for those compounds using VAMS is of interest. Itraconazole (ITZ) is a BCS class II drug with a poor solubility and was chosen as model compound in this study. The goal was to develop and validate a quantitative method for ITZ using VAMS and ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS). This was done with the intention to use it in studies testing the bioavailability of various formulations containing ITZ. For this reason, a proof of concept study was conducted in which rat blood concentrations of ITZ were determined after the administration of the commercially available Sporanox®. Moreover, the stability of ITZ absorbed on VAMS device tips was investigated. Due attention was also paid to hydroxy-itraconazole (OH-ITZ), the main metabolite of ITZ which is biologically active.

2. Materials and methods

2.1. Materials

ITZ, OH-ITZ and deuterated ITZ (ITZ-d9) (all analytical grades) were purchased from Indis NV (Aartselaar, Belgium), Toronto Research Chemicals (Toronto, Canada) and TLC Pharmaceutical Standards Ltd. (Aurora, Canada), respectively. Sporanox® (Janssen, Belgium) was purchased at a local pharmacy. Trifluoroacetic acid (TFA, HPLC grade) and sodium hydroxide were obtained from VWR International (Radnor, USA) and monosodium phosphate (Ph. Eur.) from Merck (Darmstadt, Germany). Acetonitrile (ACN), methanol (MeOH), water and formic acid (FA) of UHPLC–MS grade, and isopropanol (IPA, LC–MS grade) were supplied by Biosolve (Valkenswaard, the Netherlands). Milli-Q water (18.2 MΩ/cm resistivity) was obtained with a Millipore® system (Bradford, USA), using a 0.22 μm Millipore Millipak® filter. Whole blood was collected from male and female Wistar rats after approval by the ethical committee of the Centre Hospitalier Universitaire (CHU) of Liège.

2.2. Stock solutions and blood samples

Stock solutions of ITZ, OH-ITZ and ITZ-d9 with a concentration of 0.1 mg/mL were prepared in MeOH. Ultrasonification was used to promote the dissolution of the compounds. The solutions were aliquoted and stored at −20 °C upon further use. Working solutions with concentrations from 0.5 to 50 μg/mL were prepared by dilution of the stock solutions with MeOH. The working solutions were added to fresh rat blood to obtain blood samples which were subsequently collected with VAMS devices. After the collection of 10 μL, the blood was dried at ambient temperature for two hours.

2.3. Sample pretreatment

2.3.1. Extraction procedure

The dried blood samples were processed in order to extract ITZ and OH-ITZ. The sample preparation was adopted from Houbart et al. [5] and further optimized in order to obtain optimal and reproducible extraction recoveries for ITZ and its main metabolite. The extraction was carried out in an Ostro® 96-well sample preparation plate (Waters®, Dublin, Ireland) in order to additionally remove phospholipids. The extraction solvent (200 μL) containing the internal standard ITZ-d9 (25 ng/mL) was put into the Ostro®-plate and the tips of the VAMS were placed in the solvent to be incubated for 5 min. Subsequently, the samples were agitated for 5 min using a vortex mixer (Scientific Industries®, New York, USA). The solutions were collected in a 96-well plate (Agilent Technologies®, Santa Clara, USA) by passing it through the Ostro®-plate using a vacuum manifold of Waters®.

2.3.2. Selection of extraction solvent

The selection of the extraction solvent is of utmost importance [10]. Therefore, various solvent mixtures for the extraction of ITZ were tested in duplicate. For that purpose, a blood sample containing 2 μg/mL ITZ was prepared (Section 2.2) and extracted according to the procedure described in Section 2.3.1. The extraction recoveries were determined using a previously validated high performance liquid chromatography (HPLC) method with ultraviolet (UV) detection. An Agilent® 1100 LC System was operated using the OpenLab CDS LC ChemStation software (version C.01.05). Chromatographic separation (run time of 10 min) was performed on a GraceSmart® RP 18 column (250 mm x 4.6 mm i.d.) with particles of 5 μm. The mobile phase was composed of ACN-phosphate buffer (pH 6) in a proportion of 65:35 v/v. The flow rate was set at 1.0 mL/min and the column temperature was kept constant at 30 °C. In total, 100 μL of the extracted samples was injected and ITZ was detected at a wavelength of 263 nm. The concentration of ITZ in the extracted samples was assessed based on a calibration curve constructed with calibration standards in MeOH. The extraction recoveries were calculated by dividing the obtained concentrations by the spiked concentration of 2 μg/mL.

2.3.3. Evaporation and reconstitution

After the selection of the extraction solvent, the sample preparation was further optimized with the incorporation of an evaporation procedure followed by a reconstitution step. The solvent was evaporated under vacuum at 50 °C for 60 min using a CentriVap Concentrator of Labconco (Kansas City, USA). After evaporation, the dried samples were reconstituted in 50 μL MeOH-H_2O (50:50, v/v) and analysed with UHPLC–MS/MS.

2.4. UHPLC–MS/MS

An UHPLC–MS/MS method was optimized to quantify ITZ and OH-ITZ in rat blood. Extracted blood samples were analysed on an Agilent® 1290 Infinity LC system coupled to a 6495 triple quadrupole mass spectrometer with an Agilent® Jet Stream source and iFunnel technology. A Kinetex® F5 column (2.6 μm, 50 × 2.1 mm i.d.) connected to a Security Guard Ultra F5 precolumn (both from Phenomenex®, Torrance, USA) was used for the chromatographic separation. In the optimized UHPLC–MS/MS method, the processed samples (5 μL) were injected and analysed under gradient elution with mixing mobile phase A (0.1% FA in H_2O) and mobile phase B (0.1% FA in ACN-H_2O (95:5, v/v)) in varying ratios at an overall flow rate of 0.8 mL/min. The gradient scheme was as follows: 0.0-0.5 min, 90% A; 0.5–4.0 min, from 90% A to 0% A; 4.0-4.5 min, 0%. After each run, the system was re-equilibrated for 1 min at 90% A. During separation, the column temperature was kept constant at 40 °C. Samples were stored in the autosampler of the UHPLC-system at 6 °C. In-between runs, the inside and outside of the injector-needle and sample loop were cleaned using an injector program. The outside of the needle was washed with a mixture of MilliQ-water, IPA and MeOH in a proportion of 20:40:40, v/v/v. Subsequently, the inside of the needle and sample loop was washed with 1% TFA in MeOH.

The mass spectrometer was operated in positive ionization mode, applying the following optimized conditions. The dry gas temperature and flow were 230 °C and 11 L/min, respectively. The nebulizer pressure was set at 55 psi and the capillary voltage at 4500 V. The sheath gas temperature and flow were 400 °C and