Journal of Chromatography B, 1020 (2016) 111–119

Contents lists available at ScienceDirect

Journal of Chromatography B

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

# Simultaneous determination of itraconazole, hydroxy itraconazole, keto itraconazole and *N*-desalkyl itraconazole concentration in human plasma using liquid chromatography with tandem mass spectrometry

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ARTICLE INFO

*Article history:*
Received 27 February 2016
Received in revised form 15 March 2016
Accepted 25 March 2016
Available online 26 March 2016

*Keywords:*
LC–MS
Human plasma
Itraconazole
Hydroxy itraconazole
Keto itraconazole
*N*-desalkyl itraconazole
Drug-drug interaction

ABSTRACT

A high-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) assay was developed and validated for simultaneous determination of itraconazole (ITZ), hydroxy-itraconazole (OH-ITZ), keto-itraconazole (keto-ITZ) and *N*-desalkyl itraconazole (ND-ITZ) concentration in human plasma. One hundred and fifty microliters of human plasma were extracted using a solid-supported liquid extraction (SLE) method and the final extracts were analyzed using reverse-phase chromatography and positive electrospray ionization mass spectrometry. The standard curve range is 5–2500 ng/mL for ITZ and OH-ITZ and 0.4–200 ng/mL for keto-ITZ and ND-ITZ. The curve was fitted to a $1/x^2$ weighted linear regression model for all analytes. The precision and accuracy of the LC–MS/MS assay based on the five analytical quality control (QC) levels were well within the acceptance criteria from both FDA and EMA guidance for bioanalytical method validation. Average extraction recovery was 97.4% for ITZ, 112.9% for OH-ITZ, 103.4% for keto-ITZ, and 102.3% for ND-ITZ across their respective curve range. Matrix factor was close to 1.0 at both high and low QC levels of all 4 analytes, which indicates minimal ion suppression or enhancement in our validated assay. Itraconazole and all three metabolites are stable in human plasma for 145 days stored at −70 °C freezers. The validated assay was successfully applied to a clinical study, which has a drug-drug interaction (DDI) arm using ITZ as a cytochrome P450, family 3, subfamily A (CYP3A) inhibitor.


## 1. Introduction

Itraconazole (ITZ) is a triazole antifungal agent with a broad spectrum of activity, which is prescribed to patients with fungal infections [1,2]. It has also recently been explored as an anticancer agent for patients with basal cell carcinoma, non-small cell lung cancer, and prostate cancer [3–5]. ITZ is currently considered by regulatory agencies (US FDA and EMA) to be the CYP3A inhibitor replacing ketoconazole in clinical drug-drug interactions (DDIs) due to serious potential side effects and the risk for liver injuries associated with ketoconazole [6,7]. However, unlike ketoconazole, ITZ has not been extensively used in the clinical DDI study and the optimal DDI study has yet to be determined [8,9]. ITZ is a potent cytochrome P450, family 3, subfamily A (CYP3A) inhibitor that can cause significant DDI when co-administrated with other CYP3A substrates in the clinic. Both in vitro and in vivo studies indicate that ITZ is metabolized to form three metabolites, hydroxy-itraconazole (OH-ITZ), keto-itraconazole (keto-ITZ), and *N*-desalkyl-itraconazole (ND-ITZ) [10–12], and all three metabolites are also potent CYP3A inhibitors. However, the contribution of the metabolites to the observed clinical DDI has not been fully evaluated partly due to the lack of assay, which can be used to quantify the metabolites accurately. It has been reported that predictions of ITZ DDI by considering only ITZ CYP3A inhibition often under-estimate the DDI observed clinically [10,13–15]. One of the explanations is that circulating ITZ metabolites, such as OH-ITZ, ND-ITZ and keto-ITZ, may also contribute to the reported clinical DDI through inhibition of CYP3A. By accounting for these circulating

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http://dx.doi.org/10.1016/j.jchromb.2016.03.039
1570-0232/

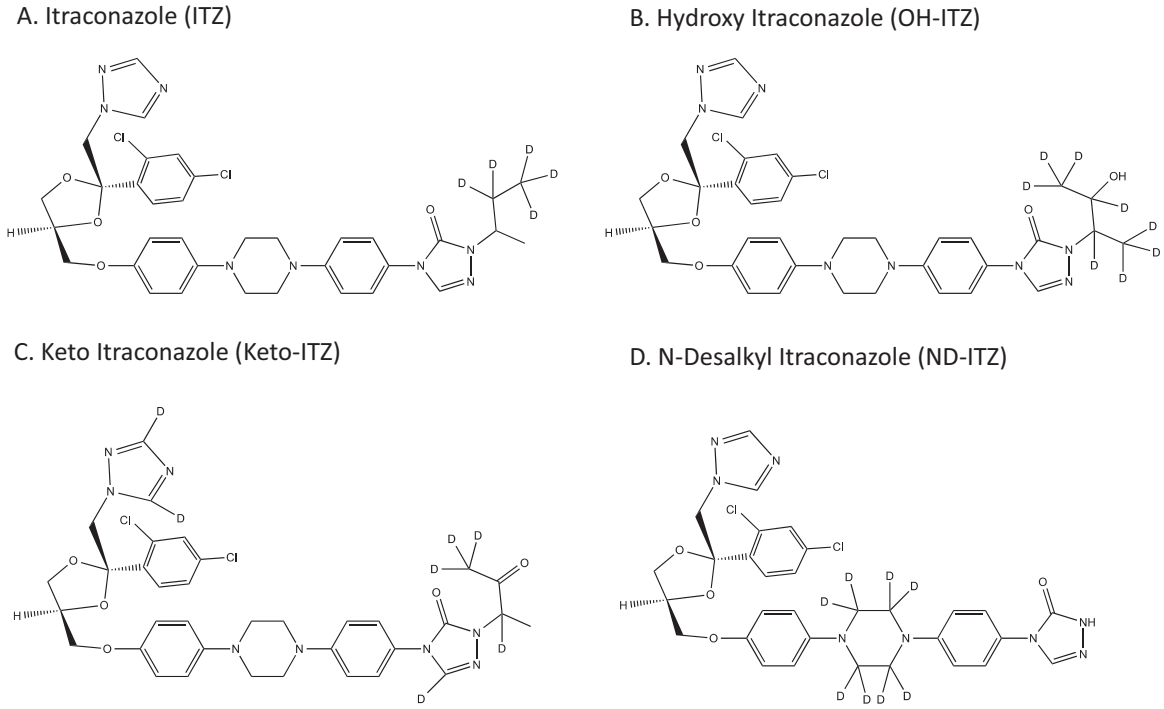


**Fig. 1.** The structures of ITZ, OH-ITZ, keto-ITZ, and ND-ITZ. D indicates the deuterium labeled position for their corresponding internal standards.

**Table 1**
Gradient program.

| Total Flow | 0.75 ml/min |
|---|---|
| Time (minutes) | Mobile Phase B (%) |
| 0.01 | 35 |
| 0.2 | 35 |
| 2.5 | 56 |
| 2.7 | 98 |
| 3.5 | 98 |
| 3.7 | 20 |
| 4.2 | 20 |
| 4.4 | 35 |
| 4.9 | 98 |
| 5.7 | 98 |
| 5.9 | 20 |
| 6.4 | 20 |
| 6.6 | 35 |
| 7.1 | System Stop |

metabolites, the prediction of ITZ DDI can potentially be significantly improved [11]. Thus, it becomes necessary to quantify the concentration of all three metabolites in addition to the parent ITZ in the clinical DDI samples, in order to further understand the mechanism and contribution of these metabolites to the clinical observed DDI. Ultimately, this will assist optimal clinical ITZ DDI study design.

Validated LC–MS assays have been reported for determining ITZ and OH-ITZ in human plasma [16–20]. However, according to the authors' knowledge, there is no published literature of a validated human plasma assay(s) for simultaneous quantitation of ITZ, and all three metabolites, OH-ITZ, keto-ITZ, and ND-ITZ. Therefore, there is a need to develop and validate such an assay per the FDA (2001) and EMA (2011) bioanalytical method validation guidance.

In this work, an LC–MS/MS assay was developed and validated for simultaneous determination of the concentration of ITZ and three inhibitory metabolites, OH-ITZ, keto-ITZ, and ND-ITZ, in human plasma. The structures of ITZ and its three metabolites are shown in Fig. 1, where the deuterium labeled positions are also indicated for the corresponding internal standard (IS). The method has been validated according to FDA and EMA guidance for bioanalytical method validation. This validated assay has been successfully applied to a clinical study, which has a DDI arm using itraconazole as a CYP3A inhibitor.

## 2. Experimental

### 2.1. Chemicals, reagents, and materials

ITZ, ND-ITZ, OH-ITZ, and keto-ITZ, ITZ-$d_5$, OH-ITZ-$d_8$, and ND-ITZ-$d_8$ were purchased from TLC Pharmaceutical Standards Ltd. (Ontario, Canada). Keto itraconazole-$d_7$ was custom synthesized by Alsachim (Illkirch-Graffenstaden, France). Formic acid (~98%), ammonium acetate, dimethyl sulfoxide (≥99.9%, DMSO), acetonitrile, and methyl tertiary butyl ether (MTBE) were purchased from Sigma-Aldrich (MO, USA). J.T. Baker methanol was purchased from VWR international (Radnor, PA, USA). Human $K_2$EDTA plasma was purchased from Bioreclamation, Inc. (Hicksville, New York, US). The analytical column, Kinetex® F5 50 × 2.1 mm 2.6 μm, was purchased from Phenomenex (Torrance, CA, USA). Isolute SLE+ supported-liquid extraction (SLE) plates were purchased from Biotage (Charlotte, NC, USA).

### 2.2. Human blood sample collection procedure

Human blood samples were collected in Vacutainer® [Becton Dickinson and Company] tubes containing $K_2$EDTA and maintained on wet ice prior to processing. After obtaining the blood sample, collection tubes were slowly inverted 8–10 times to mix the blood and anticoagulant thoroughly. Then the blood sample was centrifuged at 2000*g* for 10 min in a refrigerated centrifuge at 2–8 °C to harvest the plasma. The human plasma was transferred to a labeled sample tube and stored at −70 °C until analysis.