Supplemental Methods

Chemicals and reagents

Optima LC-MS grade acetonitrile, water and methanol were purchased from Fisher Scientific (New Jersey, USA). Analytical grade formic acid was purchased from Acros Organics (New Jersey, USA). Itraconazole and hydroxy-itraconazole were purchased from Selleck Chemicals and Toronto Research Chemicals, respectively. Posaconazole was obtained from Sigma. All other chemicals and reagents were of analytical grade and used without further purification. Blank human lung tissue matrix was procured from Bio reclamation, Inc. (New York, USA).

Preparation of stock and working solutions of analyte and internal standard

Primary stock solutions of itraconazole and hydroxy-itraconazole for the calibration curve (CC) and quality control samples (QC) were prepared (separate weighing for CC and QCs) by accurately weighing itraconazole or hydroxy-itraconazole and dissolving in sufficient dimethyl sulfoxide to yield a 200 µg/ml stock solution. Stock solutions of itraconazole and hydroxy-itraconazole were stored at -20 °C, and subsequent work stock dilution at 100 µg/ml was prepared by mixing equal amounts of itraconazole and hydroxy-itraconazole stock solutions.

Primary stock solution of the internal standard (IS) posaconazole was prepared by accurately weighing posaconazole and dissolving in chloroform to yield a 1 mg/ml main stock solution and subsequent work stock solution at 2 µg/ml was prepared using acetonitrile: water (70:30, v/v). Both main stock solution and the work stock of posaconazole were stored at -20 °C,

Extraction solvent for tissue sample was prepared by spiking 1 ml of working stock (2 µg/ml) of posaconazole into 19 ml of Methyl tert-butyl ether (MTBE) and stored at room temperature for everyday use.

Preparation of calibration curve and quality control samples for analysis of tissue samples

The method for quantitation of itraconazole and hydroxy-itraconazole had the calibrators over the range of 5 to 5000 ng/ml. Calibration curve samples were prepared by spiking 20 µl of the working stock (100 µg/ml itraconazole/ hydroxy-itraconazole) into 380 µl of blank lung tissue (blank lung tissue was prepared in the same manner as clinical tissue samples). This represented the top calibration curve point (i.e., the upper limit of quantification or ULOQ). The remaining calibration curve samples were prepared by serial dilution of the ULOQ standard in blank tissue. CC concentrations were 5, 25, 50, 100, 250, 500, 1000, 2000 and 5000 ng/ml. Quality control samples were prepared in a similar fashion by spiking 30 µl of the working stock (100 µg/ml itraconazole/ hydroxy-itraconazole) into 970 µl of blank tissue. This represented the high-quality control standard (HQC). The medium-quality control standard (MQC) and low-quality control standard (LQC) were prepared by serial dilution of the HQC standard in plasma. Spiking volume of the working standard did not exceed 5% of the matrix volume. QCs for Calibration curve were prepared at 75, 300, 1500 and 3000 ng/ml concentrations in tissue, respectively.

Tissue Sample Preparation:

Frozen lung tissue (blank) was placed on a clean and dry glass plate. The tissue was cut into small sections of approximately 2 g and then accurately weighed. The sample was placed into a 2.0 mL Eppendorf centrifuge tube, add 1 ml of water and homogenize the tissue using DPS-20 dual processing system from PRO Scientific INC., (Oxford, CT, USA). Transferred the homogenate into a 15ml conical tube and added four volume of the original tissue weight (~8ml) to keep the dilution factor as 5×.

Frozen study samples were homogenized the exact same way except the volumes were adjusted to facilitate to achieve smooth homogenate. The dilution factors were kept in consideration to back calculate the concentration later.

To 50 µl of the tissue homogenate 20 µl of 2M ammonium acetate buffer was added and briefly vortexed. 600 µl of extraction solvent spiked with IS was added to the above mix and the resultant suspension was vortexed for 30 min at 2500 rpm, followed by centrifugation at 12,000 rpm for 5 min. Five hundred microliters (500µl) of the clear supernatant, was removed, placed in a new Eppendorf tube and dried using a SpeedVac. Samples were reconstituted using 200 µl of acetonitrile:water (70:30).

HPLC operating conditions

A Shimadzu CBM-20A Nexera series LC system (Shimadzu Corporation, Kyoto, Japan) equipped with degasser (DGU-20A) and binary pump (LC-30AD) along with auto-sampler (SIL-30AC) and (CTO-30A) column oven. The autosampler was maintained at 10 °C. An injection volume of 5 µl was used and chromatographic separation was achieved using a Kinetex C18 (1.7 µm, 2.1 × 50mm) column. The mobile phase, consisting of 0.1% formic acid in water (pump A) and acetonitrile with 0.1% formic acid (pump B) used for the method. The mobile phase pumped using a gradient program at a flow rate of 0.5 ml/min into the mass spectrometer electrospray ionization chamber in positive polarity. Gradient program initiated with 10% of B and maintained for 1.0 min, then ramped to 90%B by 2.0 min and maintained at 90%B until 3.0 min, changed back to 10%B by 3.1 min and maintained until 5.0 min before getting back to initial gradient.

Mass spectrometry operating conditions

Quantitation was achieved by employing electrospray ionization in positive ion mode for the analytes using a SCIEX QTRAP 5500 mass spectrometer (Redwood, CA, USA) equipped with the ion source voltage 5500 V, Turbo V source operated at 550 ºC. The nebulizer gas, auxiliary gas, curtain gas, CAD gas were set at 50, 50, 40 psi and medium, respectively. The de-clustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 100, 55, 10, 20 V for itraconazole; 100, 49, 10, 15 V for hydroxy-itraconazole; and 100, 50, 10, 13 V for Posaconazole respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 705.4

precursor ion to the *m/z* 392.4 for itraconazole, transition pairs of *m/z* 721.3 precursor ion to the *m/z* 408.3 for hydroxy-itraconazole, and transition pairs of *m/z* 701.204 precursor ion to the *m/z* 683.3 product ion for the Posaconazole. The data obtained were processed by Analyst software™ (version 1.7.1).