Mass Spectrometry Compatible Separation of Itraconazole and Related Substances by UHPLC

INTRODUCTION

An improved analysis method is now available for itraconazole (structure shown in Figure 1A), one member of a family of triazole antifungal agents. Azoles share the same antifungal mechanism that prevents formation of the ergosterol necessary for the fungi’s cell wall. As a broad spectrum antifungal, itraconazole is approved by the United States Food and Drug Administration (FDA) for systemic mycoses, onychomycoses, blastomycosis, histoplasmosis, and aspergillusin in patients who cannot tolerate amphotericin B.

The United States Pharmacopeia (USP) analysis method for itraconazole and its related substances uses high-performance liquid chromatography (HPLC) with single wavelength UV or diode array detection (DAD).1 Tetrabutylammonium hydrogen sulfate (TBAHS) is added to the mobile phase to improve itraconazole retention and peak shape, possibly by decreasing the effect of the stationary phase's residual silanols. The analysis method for itraconazole from the China Pharmacopoeia (CP) is similar to the USP method.2 Generally, any impurity of a synthetic drug that is greater than 0.1% must have its structure elucidated. This is often accomplished with the aid of mass spectrometry (MS) and many times with liquid chromatography-mass spectrometry (LC-MS). A MS-compatible mobile phase is needed for LC-MS, and the USP and CP mobile phases for the itraconazole analysis method are not MS compatible due to the TBAHS.

Though a minor concern, quaternary ammonium salt ion-pairing agents can damage silica-based packing materials. In addition to the MS-incompatible mobile phase, the CP method for itraconazole analysis requires 50 min. Therefore, there are good reasons to try to improve the itraconazole analysis method.

A literature search reveals HPLC methods to determine the concentration of itraconazole and hydroxyitraconazole in plasma using an MS-incompatible mobile phase.3–5 Dionex published an on-line solid-phase extraction (SPE)-LC method for analysis of multiple drugs, including itraconazole, using ammonium acetate as the mobile phase buffer.6

![Figure 1. Structures of itraconazole A) and miconazole B).](image-url)
All of these methods were designed to determine itraconazole in plasma samples and were not concerned with itraconazole impurities. In pharmaceutical companies, separation and quantitation of trace amounts of impurities are usually the focus of method development. The HPLC methods reported in the literature also did not determine miconazole (structure shown in Figure 1B), which is an important reference substance for resolution control in the pharmacopeia methods.

This work demonstrates an efficient program for analyzing itraconazole and its related substances without an ion-pairing agent. The study also investigates the chromatographic behavior of itraconazole and miconazole under different diluent constitutions and/or volumes. The effect of buffer constitution and concentration on peak shape is also discussed.

The separation was performed on the Thermo Scientific Acclaim® 120 C18 Rapid Separation LC (RSLC) column and Thermo Scientific Dionex UltiMate® 3000 HPLC system with DAD detection using a mobile phase comprised of an acetate buffer, water, and acetonitrile. The analysis was completed within 15 min. The method was also compatible with MS and was executed as an LC-MS method to identify the related substances.

**EQUIPMENT**

Dionex UltiMate 3000 quaternary Rapid Separation LC (RSLC) system including:

- LPG 3400RS Pump with a 350 μL mixer
- WPS 3000TRS Autosampler with a 100 μL sample loop
- TCC-3000 Thermostatted Column Compartment (preheater not used)
- DAD-3000RS Diode Array Detector with a 13 μL flow cell

Thermo Scientific MSQ Plus™ single quadrupole mass spectrometer (MS) with an ESI source

Thermo Scientific Dionex Chromelon® 6.80 SR9 Chromatography Workstation

Mettler Toledo AL-204 analytical balance, Mettler-Toledo Instruments (Shanghai) Co., Shanghai, China

**REAGENTS AND STANDARDS**

Water, from Milli-Q® Gradient A 10

Acetonitrile (CH₃CN), HPLC grade, Fisher

Methanol (CH₃OH), HPLC grade, Fisher

Ammonium acetate (NH₄Ac), analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Formic acid, analytical grade, SCRC, China

Tetrahydrofuran (THF), HPLC grade, SCRC, China

Tetrabutylammonium hydrogen sulfate (TBAHS), HPLC grade, J&K Scientific, China

Itraconazole (99.3%), NICPBP, China

Miconazole nitrate salt, Sigma

**CHROMATOGRAPHIC CONDITIONS**

Analytical Column: Acclaim RSLC 120, C18, 2.2 μm, 2.1 × 100 mm, P/N 068982

Column Temp.: 30 °C

Mobile Phase: HAc/NH₄Ac buffer to CH₃CN gradients (details shown in figures)

Flow Rate: 0.45 mL/min

Inj. Volume: 2 or 10 µL

UV Detection: Absorbance at 225 nm

**MS-PLUS MS CONDITIONS**

Ionization Mode: ESI

Operating Mode: Positive Scan

Probe Temp.: 400 °C

Needle Voltage: 2000 V

Mass Range: 400–1000 amu

Scan Time: 0.3 sec

Cone Voltage: 80 V

Nebulizer Gas: Nitrogen at 75 psi

**PREPARATION OF STANDARDS**

**Stock Standard Solutions**

Dissolve accurately weighed 10 mg of itraconazole in 10 mL diluent (a mixture of CH₃OH and THF, 4:1, v/v).

The concentration of itraconazole is 1 mg/mL. Prepare a 1 mg/mL solution of miconazole in the same manner.

**PREPARATION OF SAMPLES**

**Itraconazole Capsule Sample**

Itraconazole capsules, made by Xian-Janssen Pharmaceutical LTD, China, were purchased from a local pharmacy.
Dissolve the accurately weighed contents of an itraconazole capsule equivalent to 50 mg itraconazole in 250 mL CH₃OH/THF mixture (4:1, v/v). (The capsule used in this work contained 100 mg of itraconazole, so half the weight of the capsule contents was used.) Sonicate to aid dissolution and filter the solution through a 0.45 µm filter (Millex®-HV) and store at room temperature.

**Itraconazole Capsule Hydrolysis Sample**

Dissolve the accurately weighed contents of an itraconazole capsule equivalent to 20 mg itraconazole (20% of the capsule contents for the sample used in this work) in 1 mL formic acid. Incubate the solution in a 60 °C water bath for 3 h. Dilute the solution with 9 mL CH₃OH/THF mixture (4:1, v/v). Store the sample at room temperature for at least 24 h before analysis.

**RESULTS AND DISCUSSION**

**Development of an Improved Itraconazole Analysis Method**

Both the itraconazole method from the CP (4.6 mm diameter column) and an improved method (2.1 mm diameter column) were used to separate itraconazole and its related substances (Figures 2A and 2B). The faster method separated at least as many itraconazole related substances as the CP method. Initially, the same sample volume and diluent used in the USP and CP methods were also used in the faster method, and the itraconazole peak exhibited fronting. The THF in the sample diluent seemed to impact peak fronting because the fronting increased when more THF was present in the diluent.

Miconazole—the resolution reference substance for itraconazole in the USP—fronted in the same manner as itraconazole when TBAHS was used in the mobile phase. In contrast, itraconazole was influenced more heavily than miconazole by the diluent constitution when NH₄Ac/HAc buffer replaced TBAHS in the solvent system. As shown in Figure 3, a 10 µL itraconazole injection in a 1:1 methanol:THF diluent led to obvious itraconazole peak fronting, whereas no fronting was observed for miconazole. When the composition of the dilute was changed to 4:1 methanol:THF, fronting was eliminated. Reducing the injection volume to 2 µL eliminated peak fronting even when 1:1 methanol:THF was used.

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**Figure 2. Chromatograms of an itraconazole standard using A) method from the CP and B) modified method with a shorter run time.**

**Figure 3. Overlay of chromatograms of itraconazole and miconazole using diluents as 1) CH₃OH and THF (4:1, v/v) with 2 µL injection; 2) CH₃OH and THF (4:1, v/v) with 10 µL injection; 3) CH₃OH and THF (1:1, v/v) with 2 µL injection; and 4) CH₃OH and THF (1:1, v/v) with 10 µL injection.**
Effect of pH on Retention Times and Peak Shapes of Itraconazole and Miconazole

The pH of the mobile phase was adjusted by changing the ratio of HAc to NH₄Ac. HPLC analysis was performed using the following ratios of HAc to NH₄Ac: 19:1, 15:5, 10:10, 5:15, and 1:19. The retention time of miconazole decreased significantly when the solvent system was more acidic (Figure 4). The asymmetry of miconazole peak increased as retention time decreased. In contrast, neither itraconazole retention time nor peak shape were affected significantly by mobile phase pH.

Figure 4. Comparison of retention times of itraconazole and miconazole at different pH values (buffer concentration: 20 mM). The corresponding pH values of the HAc/NH₄Ac buffer are 5.92, 5.15, 4.54, 4.25, and 3.26.

Effect of Buffer Concentration on Peak Shape of Itraconazole and Miconazole

One objective of this work was to develop a method that removed TBAHS from the mobile phase. Initially this was attempted using only water and acetonitrile in bottles A and B, respectively. Without TBAHS, the miconazole peak disappeared whereas itraconazole remained intact. A gradually increasing concentration of buffer (NH₄Ac/HAc) was added to the mobile phase to improve miconazole peak shape. Miconazole, with its basic moiety as imidazole, is a medium-strong base. In contrast to itraconazole (which is a very weak base, considering its 1,2,4-triazole structure), miconazole may have stronger interaction with silanol residues. This explains why a higher concentration of TBAHS is needed to keep miconazole peak shape, compared to itraconazole. TBAHS can decrease the effect of silanol residues by either TBA–silanol interaction or the acidic effect of the HSO₄⁻ to inhibit silanol proton dissociation. As shown in Figures 5A and 5B, the buffer concentration significantly affected the miconazole peak shape. A low buffer concentration of 0.1 mM was enough to keep the itraconazole peak intact whereas the miconazole peak shape was distorted.
Separation of Itraconazole and Its Related Substances

After establishing the improved method that eliminated the ion-pairing agent, an itraconazole standard, a miconazole standard, an itraconazole acid hydrolysis sample, and a commercial itraconazole capsule were analyzed (Figure 6A). The negative sloping baseline is attributed to the different absorption coefficient of ammonium acetate buffer (bottle A) and acetonitrile (bottle B) at 225 nm. The USP uses miconazole as a resolution control whereas the CP does not have a similar resolution control. As an alternative for resolution control, itraconazole was intentionally acid hydrolyzed to generate impurities that can be used as controls to judge resolution. For comparison, the same samples were also analyzed by the method with the ion-pairing agent (Figure 6B).

Sample Analysis by LC-MS

The USP describes seven impurities in the itraconazole standard. They are the 4-methoxy derivative, the 4-triazolyl isomer, the propyl analog, isopropyl analog, an itraconazole epimer, the n-butyl isomer, and the didioxolanyl analog with m/z of 408, 705, 691, 705, 705, and 960 respectively. As shown in Figure 7, two peaks with m/z of 705.1 were observed in itraconazole by analysis in the total ion chromatogram (TIC) mode. In the more sensitive selected ion monitoring (SIM) mode, four peaks with m/z of 705.1 were observed, which correlated to itraconazole, the 4-triazolyl isomer, the epimer, and the n-butyl isomer. Also in TIC mode, two peaks with m/z of 691.1 were found which matched perfectly with the propyl and isopropyl analogs of itraconazole.
All USP-indicated m/z values were observed using the LC-MS method in SIM mode. Due to the abundance of $^{37}$Cl as 32.5% of $^{35}$Cl, a specific isotopic MS distribution pattern was expected for itraconazole, which contains two chlorines. Theoretically, the height of the 707.1 peak would be 65% of the 705.1 peak, and the 709.1 peak height would be 10.5% of the 705.1 peak. This prediction was fully confirmed by the data shown in Figure 8.

CONCLUSION

This application replaces a lengthy HPLC method that includes an ion-pairing agent with an efficient MS-compatible method that saves valuable time and resources in the analysis of itraconazole and its related substances. The Dionex Acclaim RSLC C18 column exhibits similar separation ability for itraconazole-related substances using NH$_4$Ac/HAc buffer in mobile phase as when using TBAHS. Sample diluent constitution, volume, solvent pH, and solvent ionic strength affect the chromatography of either itraconazole, miconazole, or both; therefore, these parameters were optimized to yield the best chromatography for both compounds. Use of LC-MS detected all the impurities indicated by USP including isomers having the same molecular weight.

REFERENCE