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# Microassay for determination of itraconazole and hydroxyitraconazole in plasma and tissue biopsies

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**Abstract**

A simple, rapid and sensitive method for the extraction and HPLC analysis of itraconazole and hydroxyitraconazole in tissue and plasma or serum is described. Tissue (5–100 mg) and plasma (0.1 ml) underwent a simple extraction into methanol. Chromatography was performed on a Novapak $C_{18}$ column using a mobile phase of water–acetonitrile–diethylamine (42:58:0.05, v/v), pH 2.45, with a flow-rate of 1.5 ml/min. Fluorescence was measured at excitation 260 nm and emission 365 nm. The procedure produced a linear curve for the concentration range 10–1000 ng/ml. The development of the assay produced accurate, rapid repeatable results for both tissue and plasma or serum. © 1997 Elsevier Science B.V.

*Keywords:* Itraconazole; Hydroxyitraconazole

## 1. Introduction

Itraconazole is a new generation triazole antifungal drug which has been shown to be highly effective in curing fungal infections following oral administration in humans [1–3] and animals [4–6]. It has both in vitro and in vivo activity against a broad spectrum of fungal agents such as yeasts (*Candida* and *Cryptococcus*), fungi (*Aspergillus*) and dimorphic fungi (*Histoplasma*, *Blastomyces* and *Sporothrix*) [7]. Itraconazole has been shown to be more potent than ketoconazole, with fewer side effects in various clinical trials in humans and other animals. Unlike ketoconazole, [8] it has minimal hepatotoxic side effects and has no effect on the synthesis of androgens, estrogens or glucocorticoids [9–12].

Fungal infections, particularly *Aspergillus* and *Candida*, can result in serious systemic disease and/or death. These fungal infections often occur in patients having liver and/or kidney dysfunction. The use of an antifungal drug that does not further compromise these patients is extremely important in their care. Pharmacokinetic studies are necessary to determine the concentration and dosing regimen required for clinical success.

Itraconazole has been assayed by microbiological procedures, but the levels of detection are inadequate for therapeutic monitoring [3,13–15]. These procedures are not specific since they determine total antifungal activity that may also include a contribution from active metabolites and other antifungal drugs [14]. Recently developed high-performance

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liquid chromatography (HPLC) methods [13,16–19] require 0.5–2 ml of plasma or serum and use time consuming liquid–liquid [13,17] or column extractions [18] or have an inadequate level of detection [16]. There are only a few methods involving the extraction of itraconazole from tissue [17,20,21], all of which involve lengthy liquid–liquid extractions.

This article describes a rapid and efficient clean-up of small plasma or serum and tissue samples using HPLC analysis. The serum or plasma samples were analyzed using a modification of Badcock's [19] method for analysis of itraconazole. This procedure allows for rapid analysis that may facilitate the evaluation of therapeutic efficacy of dosage regimens.

## 2. Experimental

### 2.1. Reagents and standards

Methanol and acetonitrile were "HPLC" grade (Burdick and Jackson Laboratories, Muskegon, MI, USA) while dimethylamine and diethyl sulfoxide were analytical reagent grade (Sigma Chemical, St. Louis, MO, USA). Itraconazole (R51211) (±)-*cis*-4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-pipera-zinyl]phenyl]-2,4-dihydro-2(1-methylpropyl)-3H-1,2,4-triazol-3-one and the internal standard (R51012) *cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-5-methyl-2-(3-methylbutyl)-3H-1,2,4-triazol-3-one were purchased from Janssen Research Diagnostics, (Flanders, NJ, USA). The hydroxyitraconazole was a gift from Janssen Research Foundation (Beerse, Belgium).

Stock standard solutions of itraconazole (25 and 1 μg/ml) and the metabolite (20 and 1 μg/ml) in methanol were prepared and stored at 4°C. The solutions were stable for six months. Working standards were prepared fresh daily by dilution of stock standards. Stock standard solutions of the internal standard (50 and 12.5 μg/ml) were prepared in methanol and stored at 4°C. These solutions were also stable for six months.

### 2.2. Apparatus

The analytical system consisted of a 600E solvent delivery system, a model 700 WISP autosampler, a RCM 8 mm×10 cm cartridge holder equipped with a 8 mm×10 cm Novapak $C_{18}$ cartridge (4 μm particle size) and a Novapak Guard-Pak precolumn insert, a model 470 fluorescence detector (Waters, Milford, MA, USA) and an NEC Powermate computer (NEC, Foxborough, MA, USA).

### 2.3. Chromatography

The mobile phase was an isocratic mixture of water–acetonitrile–diethylamine (42:58:0.05, v/v) at a pH of 2.45. The pH was adjusted with 85% phosphoric acid. It was prepared fresh daily using double-distilled, deionized water, filtered (0.22 μm) and degassed before use. The flow rate was 1.5 ml/min, column temperature was ambient and fluorescence was measured at excitation 260 nm and emission 365 nm. All chromatograms were obtained with the following fluorometric conditions; gain 100×, attenuation 1 and filter time constant 0.5 s.

### 2.4. Tissue extraction procedure

The tissue was weighed (25 mg) placed in 16× 100 ml disposable test tubes and homogenized in 1 ml of methanol using a polytron homogenizer (Brinkman Instruments, Westbury, NY, USA). The internal standard (50 μl of a 12.5 μg/ml solution) was added to the homogenate and vortexed. Samples were placed in the centrifuge for 15 min at 833 *g*. The supernatant was transferred to a clean test tube and evaporated to dryness under nitrogen. The residue was then reconstituted in 300 μl methanol and a 190 μl aliquot was injected onto the liquid chromatograph.

### 2.5. Plasma or serum extraction procedure

Previously frozen plasma or serum samples were thawed and vortexed before use. Prior to addition of plasma or serum (100 μl), appropriate volumes (10–1000 μl) of the standards (1 μg/ml) were evaporated under nitrogen. The internal standard, R51012 (50 μl of a 12.5 μg/ml solution) was added and

vortex-mixed. Methanol (300 μl) was added to each sample and vortex-mixed for 60 s; then centrifuged at 833 *g* for 5 min. A 190 μl aliquot was injected onto the liquid chromatograph.

## 3. Results

A representative blank chromatogram for extracted tissue is shown in Fig. 1A, with a peak that appears at 11 min. The chromatogram in Fig. 1B is an avian tissue sample (liver) collected 24 h after a 5 mg/kg dose of itraconazole. Retention times for hydroxyitraconazole, itraconazole and the internal standard were 7.40, 14.10 and 18.50 min, respectively. The endogenous peak did not interfere with the elution of the triazoles. A blank chromatogram of extracted plasma is shown in Fig. 2A. Fig. 2B is a chromatogram of avian plasma collected 24 h after administration of a 5 mg/kg dose of itraconazole. Hydroxyitraconazole, itraconazole and internal standard retention times were 7.00, 13.50 and 17.90 min, respectively. Endogenous plasma components did not interfere with the elution of itraconazole, the metabolite, or the internal standard.

The method for tissue analysis produced a linear curve for the concentration range used (10–1000 ng/mg) for both itraconazole and its metabolite, with the correlation coefficients ranging from 0.997 to 0.999 for itraconazole and 0.994 to 0.999 for hydroxyitraconazole. Replicate analyses performed on the same day for tissues spiked with specific concentrations of itraconazole showed coefficients of variation (C.V.) to be 1.8% for 75 ng/mg, 0.6% for 350 ng/mg, and 0.7% for 750 ng/mg while the metabolites' C.V. were 4.2, 2.5 and 0.9% for the same concentrations (Table 1). Day-to-day variability for tissue replicates are shown in Table 2. The mean recoveries of itraconazole from tissue were 89, 91, 90, 87, 88, 86, 87 and 93% for 10, 25, 50, 100, 250, 500, 800 and 1000 ng/mg. The mean recoveries for the metabolite were 86, 84, 85, 90, 89, 90, 79 and 92% for 10, 25, 50, 100, 250, 500, 800 and 1000 ng/mg. The detection limit for itraconazole in tissue was 5 ng/mg. This would represent a peak approximately three times baseline noise.

Correlation coefficients for the plasma curve (10–1000 ng/ml) ranged from 0.998 to 0.999. Results of

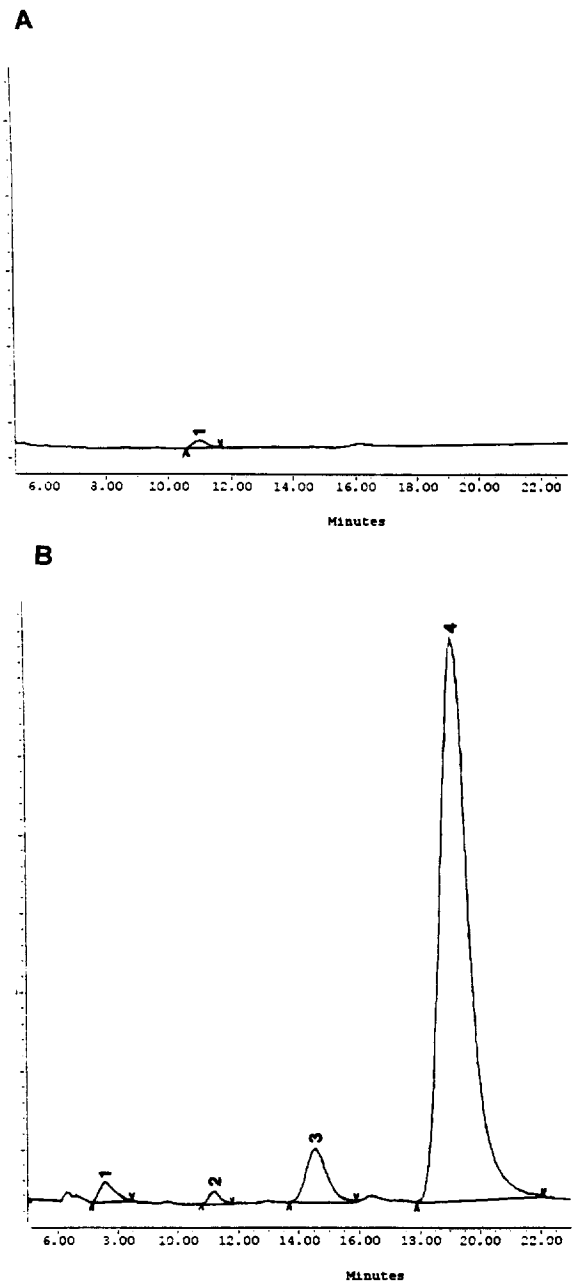


Fig. 1. (A) Chromatogram of extracted tissue with no drug added. Peak 1 results from endogenous tissue components. (B) Chromatogram of an avian tissue sample collected 24 h after administration of 5 mg/kg itraconazole. Peaks: 1=hydroxyitraconazole (30 ng/mg); 2=endogenous tissue components; 3=itraconazole (60 ng/mg); 4=internal standard (R51012).

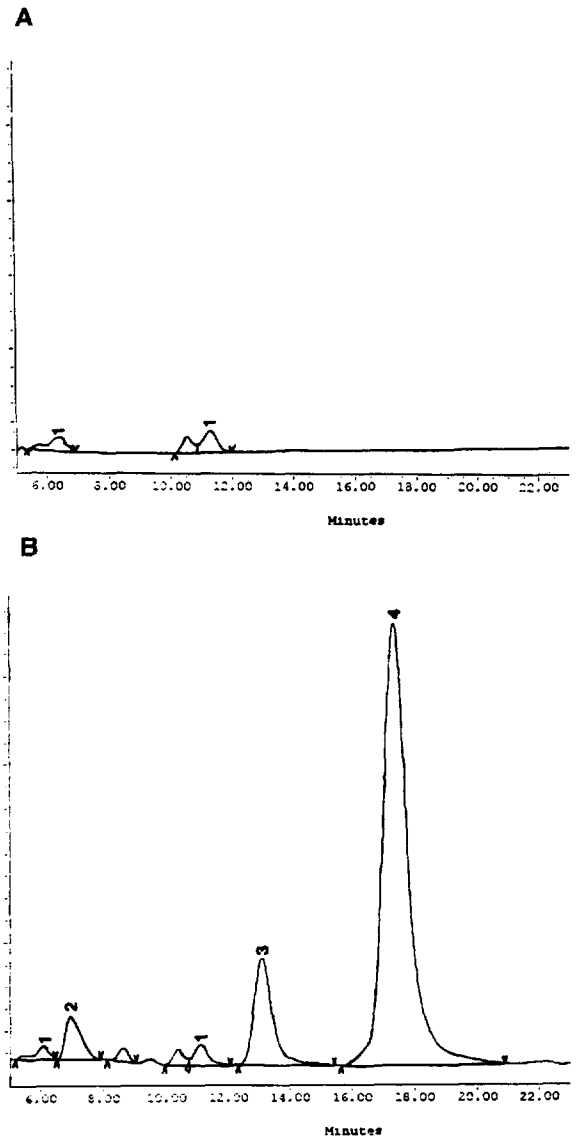


Fig. 2. (A) Chromatogram of extracted plasma with no drug added. Peak 1 results from endogoenous plasma components. (B) Chromatogram of an avian plasma sample collected 24 h after administration of 5 mg/kg itraconazole. Peaks: 1=endogenous plasma component; 2=hydroxitraconazole (62 ng/ml); 3= itraconazole (175 ng/ml); 4=internal standard (R51012).

between-day replications for plasma are shown in Table 3. The mean recoveries of itraconazole from plasma were 90, 98, 82, 90, 89, 84, 86 and 95% for 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml. The mean recoveries for the metabolite were 89, 94, 87, 90, 83, 86, 87 and 87% for 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml. The detection limit for itraconazole in serum and plasma was 5 ng/ml. This represented a peak approximately three times baseline noise.

## 4. Discussion

To be useful for both pharmacokinetic investigations and therapeutic drug monitoring, a method must be simple and rapid as well as sensitive and reproducible. Although dosage regimens for many antimicrobial agents are frequently designed on the basis of blood concentrations, therapeutic efficacy is ultimately a result of drug concentrations at the site of infection in tissues. Knowledge of tissue concentrations is particularly important for lipophilic drugs, such as itraconazole, that achieve higher tissue concentrations for a longer duration of time compared to concentrations in blood. It is often impossible to remove large amounts of tissue or blood from patients or experimental animals; therefore, an accurate reproducible procedure for small tissue biopsies and microliter-blood volumes is often needed. The procedure developed here allowed the analysis of itraconazole in tissue biopsies weighing between 5–100 mg and plasma or serum volumes less than 0.15 ml.

The detection limits and recoveries for both plasma and tissue samples are equal to or better than existing methods for extraction and analysis of itraconazole and hydroxyitraconazole. The use of R51012 as the internal standard corrects for intra- and inter-assay variability in the extraction and chromatography steps. The differences in the retention times between plasma and tissue samples are due to the different matrices of the sample. Biological samples are complex mixtures composed of a wide variety of proteins, carbohydrates, lipids, etc.. Sample mixtures separate as a result of different components adhering to or diffusing into packing particles, therefore changes in mixtures due to different matrices can cause changes in the elution of samples. It should also be noted that after hundreds of injections as the column ages there is a decrease in plate count and the peaks do tend to broaden.

HPLC procedures using extractions of itraconazole

Table 1
Intra-assay precision for itraconazole and hydroxyitraconazole tissue standards ($n=4$)

| Concentration added (ng/mg) | Itraconazole concentration measured (mean±S.D.) (ng/mg) | Hydroxyitraconazole concentration measured (mean±S.D.) (ng/mg) |
|---|---|---|
| 75 | 79±1 | 75±3 |
| 350 | 354±2 | 350±9 |
| 750 | 756±5 | 755±7 |

S.D.=Standard deviation.

from plasma/serum described by Warnock et al. [13], Woestenborghs et al. [17] and Remmel et al. [18] require 0.5 ml–2.0 ml of sample and use time consuming liquid–liquid or column extractions. Bahair [16] used a salting-out procedure and had only a 250 ng/ml level of detection. Badcock's [19] procedure used expensive reaction vials and had 6 ng/ml level of detection in plasma with a very large injection volume and it did not detect the metabolite. The few methods used for the extraction of itraconazole from tissue [17,20,21] involved lengthy liquid–liquid extractions. Heykants and colleagues [22] have quantitated the metabolite hydroxyitraconazole using heptane–isoamyl alcohol extraction with the lower limit of detection being 2–5 ng/ml. Our procedure eliminates the liquid–liquid and column extractions for both plasma/serum and tissue samples, is accurate for 5–100 mg tissue and 0.1 ml plasma/serum samples, and allows quantitation of the active metabolite. This method has been used in pharmacokinetic studies [23–25].

This procedure was developed for samples of small volumes. An equal or greater volume of deproteinizing agent is generally required for analysis of a drug in a biological sample [26,27]; therefore, increased amounts of methanol may be required for volumes of biological samples larger than used in this study. Injection volumes of up to 250 μl of

Table 2
Inter-assay precision for itraconazole and hydroxyitraconazole tissue standards ($n=4$)

| Concentration added (ng/mg) | Concentration measured (ng/mg) | Coefficient of variation (%) |
|---|---|---|
| 10 | 8 | 8.3 |
| 25 | 22 | 8.1 |
| 50 | 45 | 9.3 |
| 100 | 86 | 7.9 |
| 250 | 220 | 3.9 |
| 500 | 433 | 5.3 |
| 800 | 700 | 5.6 |
| 1000 | 933 | 8.9 |
| 10 | 8 | 6.6 |
| 25 | 21 | 8.1 |
| 50 | 43 | 1.5 |
| 100 | 91 | 7.3 |
| 250 | 222 | 11.9 |
| 500 | 451 | 2.3 |
| 800 | 635 | 1.6 |
| 1000 | 922 | 6.7 |

Table 3
Inter-assay precision of itraconazole and hydroxyitraconazole for plasma standards ($n=4$)

| Concentration added (ng/ml) | Concentration measured (ng/ml) | Coefficient of variation (%) |
|---|---|---|
| 10 | 9 | 9.1 |
| 25 | 24 | 10.0 |
| 50 | 41 | 6.6 |
| 100 | 90 | 4.0 |
| 250 | 217 | 4.6 |
| 500 | 421 | 0.5 |
| 800 | 700 | 7.0 |
| 1000 | 900 | 7.4 |
| 10 | 9 | 7.7 |
| 25 | 24 | 6.5 |
| 50 | 41 | 9.2 |
| 100 | 89 | 7.1 |
| 250 | 208 | 1.8 |
| 500 | 432 | 2.8 |
| 800 | 694 | 1.8 |
| 1000 | 875 | 5.0 |

methanol should not interfere with analysis of itraconazole and its metabolite [19]. Modification of Badcock's [19] procedure was necessary to identify both parent drug and metabolite in small sample volumes.

In conclusion, a rapid, sensitive and clinically useful HPLC procedure has been developed for analysis of itraconazole and its active metabolite, hydroxitraconazole, in microplasma or serum samples and small tissue biopsies.

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