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Rapid and sensitive high performance liquid chromatographic method for the determination of itraconazole and its hydroxy-metabolite in human serum[1]

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Abstract

Published high performance liquid chromatographic (HPLC) methods for the determination of itraconazole (ITZ) in biological matrices are labor intensive, extraction-based procedures which operate at a pH approaching the limit of column tolerance, and unless modified, cannot measure its hydroxy-metabolite (OH-ITZ). A protein precipitation-based method requiring no solvent extraction and utilizing a base-deactivated C18 analytical column to minimize peak tailing is described herein. Calibration curves for OH-ITZ and ITZ were linear from 25–1500 ng ml^{-1} ($r^2 \geq 0.999$). Intra-assay relative standard deviations (R.S.D.) were below 12%. Inter-assay R.S.D. were below 14%. This method provides a rapid means for the accurate and precise determination of both OH-ITZ and ITZ concentrations in human serum.

Keywords: Itraconazole; Hydroxyitraconazole; High performance liquid chromatography; Base-deactivated analytical column; Therapeutic drug monitoring; Antifungals

1. Introduction

Itraconazole, (ITZ) (±)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]-phenyl]-2,4-dihydro-2-(1-methylpropyl)-3*H*-1,2,4-triazol-3-one) (Fig. 1A.) is an oral triazole antifungal agent used for the treatment or prophylaxis of infections caused by species of *Aspergillus*, *Histoplasma*, and *Blastomyces* in immunocompetent and immunocompromised adult patients. Following absorption, ITZ undergoes extensive metabolism via oxidative pathways. Studies in adults have established that the pharmacokinetic profile of ITZ is complex [1–10]. The absorption of ITZ is maximal in an acidic intragastric environment and, in the presence of food, it is markedly enhanced [1–3]. ITZ is slowly absorbed

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from the gastrointestinal tract, and exhibits dose dependent elimination. ITZ metabolism to a large number of metabolites including a bioactive hydroxy-metabolite, hydroxy-itraconazole (OH-ITZ) (Fig. 1B), occurs via a saturable cytochrome P-450 (CYP) -mediated process [1,2]. Therapeutic drug monitoring of ITZ is necessary for several reasons. First, there is significant variation in serum ITZ concentrations (C_{ITZ}) in a variety of patient populations [2–10]. Second, similar to other azole antifungal agents, ITZ is a substrate and inhibitor of the CYP subfamily 3A (CYP3A). Consequently, a variety of specific and non-selective inducers of hepatic and intestinal isoforms of CYP3A such as rifampin, may induce its metabolism [11]. Lastly, recent studies have suggested a correlation between ITZ serum concentrations and therapeutic or prophylactic efficacy [12,13].

Several biologic and high performance liquid chromatographic (HPLC) assays for the analysis of ITZ in serum and tissue have been described [1,14–24]. There are several disadvantages associated with the biologic assays that limit their usefulness in therapeutic drug monitoring of ITZ. The biologic assays are nonspecific and are unable to distinguish metabolite from parent compound. More significantly, OH-ITZ is bioactive, and contributes 4 to 6 times the activity of ITZ in bioassays [25]. Thus, because OH-ITZ significantly contributes to the bioactivity of ITZ, these assays overestimate the amount of bioactive drug. Secondly, these methods lack adequate precision and sensitivity for therapeutic drug monitoring [15]. Compared to the biologic methods, the HPLC methods are more sensitive [1,14,16,17,19–24]. However, the selectivity of most of the HPLC methods for OH-ITZ is undetermined. To date three reports have described the determination of OH-ITZ [1,19,24]. However, only two reported the analytical validation of their method [19,24]. Sample preparation for most of the HPLC methods is labor intensive requiring either a multi-step liquid–liquid, or solid-phase extraction procedure [1,14,19,20,22–24]. Furthermore, because ITZ is a weak base, many of these methods require that amines (i.e. diethylamine, triethylamine) be added to the mobile phase to prevent peak tailing. Because these methods utilize mobile phase amine-

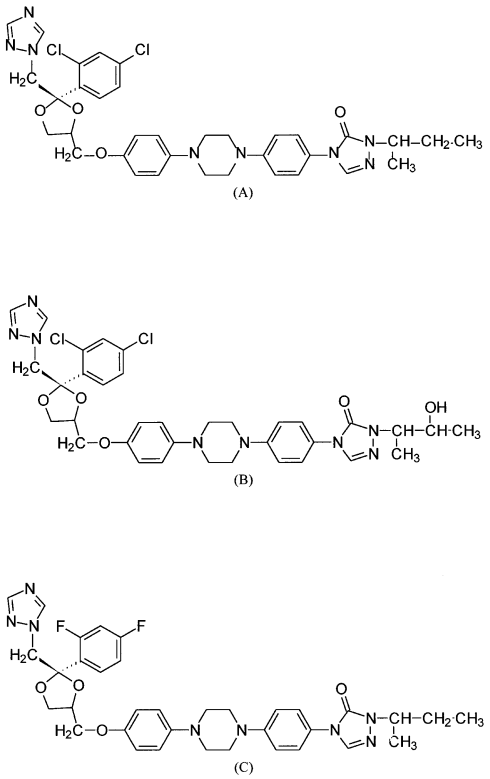

Fig. 1. Chemical structures of (A) itraconazole (ITZ), (B) hydroxy-itraconazole (OH-ITZ), (C) sapraconazole (SAP).

modifiers, they often operate at a pH approaching the limit of column tolerance [17,19,21,24]. In this report, we describe a rapid protein precipitation method for analyzing both ITZ and OH-ITZ in human serum that requires no solvent or solid-phase extraction and utilizes a base-deactivated C18 analytical column to improve peak symmetry. In addition to its effect on peak symmetry, implementation of a base-deactivated column precludes the need for mobile phase modifiers which, in turn, prolongs column life.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Burdick Jackson, (Muskegon,

MI). Analytical grade mono- and dibasic potassium phosphate, barium hydroxide and zinc sulfate heptahydrate, were purchased from Sigma, (St. Louis, MO). A methanolic reference solution containing 20 μg ml^{-1} of ITZ and OH-ITZ each was obtained from Janssen Research Foundation (Beerse, Belgium). Sapraconazole, (SAP) ($\pm$)-*cis*-4-[4-[4-[4-[[2-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-olan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3*H*-1,2,4-triazxol-3-one, (Fig. 1C.), was obtained as an internal standard from Janssen Biotech, NV (Olen, Belgium). Standard methanolic solutions corresponding to 1 μg ml^{-1} or 0.25 μg ml^{-1} were prepared for ITZ and OH-ITZ. A methanolic standard solution corresponding to 2 μg ml^{-1} was prepared for SAP.

2.2. Chromatographic system and conditions

HPLC analysis was carried out using a Shimadzu (Columbia, MD) HPLC system composed of the following: a model LC10AD solvent delivery module, a model SIL10-A automatic sample injector (100 well capacity) equipped with a 50 μl stainless steel sample loop, a model SCL-10 system controller module, a model CTO-10A column oven, and a CR-501 Chromatopac recorder/integrator set at an attenuation of 2. Column effluent was monitored by ultraviolet absorbance detection (263 nm) using a Waters Associates (Milford, MA) model 481 LC UV absorbance detector. The analysis was performed on a analytical C-18 base-deactivated (250 $\times$ 4.6mm I.D., 5 μm particle size) Alltech Alltima® column (Alltech Associates, Deerfield, IL), heated to 37°C and protected by a C-18 (7.5 $\times$ 4.6 mm I.D., 5 μm particle size) Alltech Alltima® guard column (Alltech Associates, Deerfield, IL). The mobile phase consisted of acetonitrile, 0.05 M phosphate buffer (pH 6.7), and methanol (47:45:8 v/v). The phosphate buffer was prepared using doubly distilled water. The mobile phase was vacuum filtered through a HVLP 0.45 μm filter (Millipore, Bedford, MA) and degassed under vacuum with sonication. The mobile phase was pumped at a rate 1 ml min^{-1} and continuously sparged with helium.

2.3. Calibration procedure

Using the methanolic standard solutions, samples of drug-free human plasma in clean borosilicate conical test tubes were spiked with ITZ and OH-ITZ at concentrations ranging from 20 ng ml^{-1} to 1.5 μg ml^{-1}, and SAP at fixed concentrations of 500 ng ml^{-1}. All calibration samples were taken through the precipitation procedure described below. Peak area ratios (OH-ITZ/SAP, ITZ/SAP) were used for quantitative computations. Calibration curves were calculated by linear regression analysis using a commercial software package (Microsoft Excel® Analysis ToolPak, Microsoft, USA and GreyMatter International, Cambridge MA).

2.4. Precipitation procedure

Vaildation and quality control (QC) samples were prepared via serial dilution of the OH-ITZ and ITZ 1 μg ml^{-1} standard solution in clean borosilicate conical test tubes and 62.5 μl of SAP (2 μg ml^{-1}) was then added to each tube. All conical test tubes were placed in a 60°C water bath and the organic solvent was evaporated to dryness under a stream of nitrogen. Once evaporated, 250 μl of drug-free serum was pipetted into each conical test tube to give serum standards of 0.02, 0.025, 0.050, 0.100, 0.250, 0.500, 1.0 and 1.5 μg ml^{-1} and a final SAP concentration of 500 ng ml^{-1}. Three separate QC samples containing, 0.208, 0.448, and 0.778 μg ml^{-1} of OH-ITZ and ITZ each were prepared. The conical test tubes were then vortex-mixed for 15 s. To each conical test tube 50 μl of 0.3N $BaOH_2$ and 50 μl of 0.4 N $ZnSO_4 \cdot 7H_2O$ were added and vortex-mixed for 15 s. One ml of acetonitrile was then added to each tube and the contents were vortex-mixed for 1 min. Each tube was then centrifuged at 3521 $\times$ *g* for 15 min. The supernate was transferred to clean tubes and the organic solvent evaporated to dryness at 60° C under a stream of nitrogen. Samples were then reconstituted with 250 μl mobile phase and vortex-mixed for 30 s. The resulting solutions were then transferred to separate microcentrifuge tubes and centrifuged at 12 500 $\times$ *g* for 10 min. Supernates were transferred to

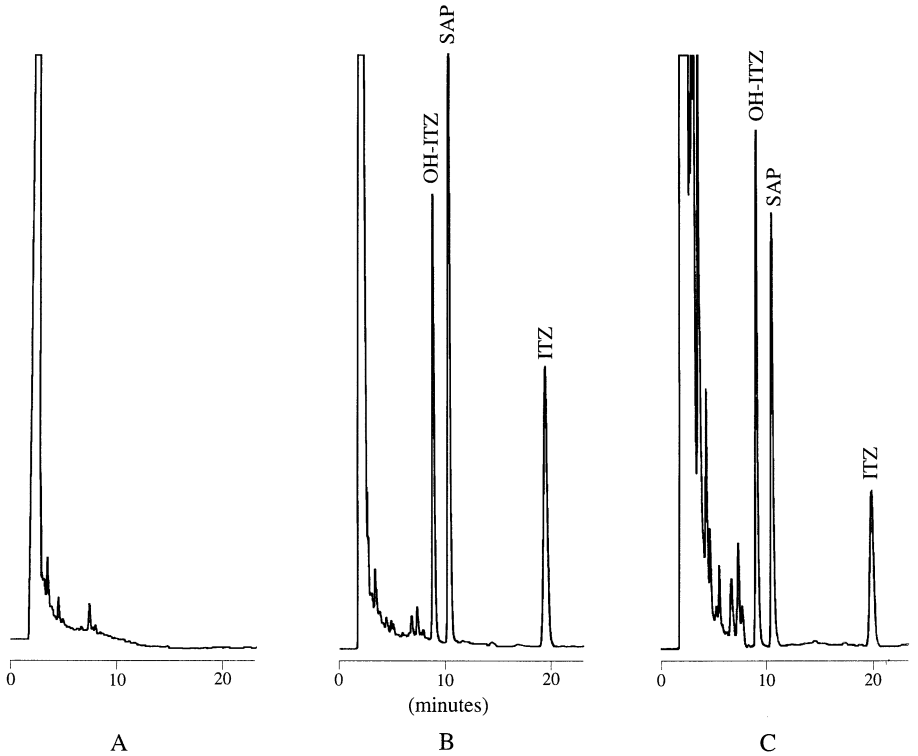

Fig. 2. Typical chromatograms using described method. (A) Blank human serum (AUFS = 0.01). (B) Human serum spiked with 250 ng ml^{-1} ITZ and OH-ITZ, and 500 ng ml^{-1} SAP (AUFS = 0.01). (C) A predose serum sample obtained at steady-state from a bone marrow transplant recipient following 21 days of administration, spiked with 500 ng ml^{-1} SAP and containing 363 ng ml^{-1} ITZ and 721 ng ml^{-1} OH-ITZ (AUFS = 0.02). For chromatographic conditions see Section 2. OH-ITZ, hydroxy-itraconazole; ITZ, itraconazole; SAP, sapraconazole; AUFS, absorbance units full scale.

injection vials and injected onto the analytical column.

2.5. Method validation

The absolute recoveries of ITZ and OH-ITZ were calculated by dividing the slope of the calibration standards in serum by the slope of the calibration standards in mobile phase. Peak area ratios (ITZ/SAP and OH-ITZ/SAP) were used for quantitative computations. Accuracy and precision of the method were determined by replicate analysis of seven known concentrations equally divided over the calibration curve. Inter- and intra-day accuracy was expressed as percentage deviation from the spiked value using the following equation:

$$\text{Accuracy: } \%Error = \frac{(C_{\text{mean obs}} - C_{\text{spiked}})}{C_{\text{spiked}}} \times 100$$

where $C_{\text{mean obs}}$ is the mean observed concentration for each standard, and C_{spiked} is the spiked theoretical concentration. Inter- and Intra-day precision of the method was expressed as the relative standard deviation (R.S.D.) of the mean observed concentration for each standard. The lower limit of quantitation was defined as the concentration of the lowest standard in the ana-

Table 1
Concomitant medications which do not interfere with itraconazole or hydroxy-itraconazole chromatography

Acyclovir	Cytarabine	Immune globulin	Penicillin G
Allopurinol	Dexamethasone	Granisetron	Phenoxymethylpenicillin
Amphotericin B	Erythropoietin	Leucovorin	Phenytoin
Antityhmocyte globulin	Fluconazole	Mesna	Piperacillin-tazobactam
Busulfan	Foscarnet	Methotrexate	Prednisone
Cefpodoxime-proxetil	Ganciclovir	Methylprednisolone	Ranitidine
Cephalexin	GCSF	Metronidazole	Thiotepa
Ciprofloxacin	Glutamine	Nizatidine	Trimethoprim
Cyclosporine	Heparin	Octreotide acetate	Sulfamethoxazole
Cyclophosphamide	Imipenem-cilistatin	Ondansetron	Vancomycin

lytical run (intra-day) which was quantitated with a definite level of certainty (precision $\leq 15\%$).

3. Results and discussion

Fig. 2 depicts representative chromatograms of blank human serum, human serum spiked with 250 ng ml^{-1} of ITZ and OH-ITZ, and a steady-state predose serum sample obtained from a bone marrow transplant recipient following 21 days of administration. Retention times of OH-ITZ, SAP and ITZ were approximately 9.5, 11.0 and 21.0 min, respectively. There was no interference from endogenous serum components. In addition, there has been no chromatographic interference of ITZ nor OH-ITZ by concomitant medications in patient samples analyzed to date. Medications exhibiting no evidence of chromatographic interference are listed in Table 1.

Absolute recoveries of OH-ITZ and ITZ were 92.9 and 93.3%, respectively, over the entire concentration range. The method was shown to be linear over the concentration range of 0.020 to 1.5 μg ml^{-1} in human serum for both OH-ITZ and ITZ. OH-ITZ had a mean slope of 0.0019 (S.D. = 0.0001, $n = 6$), and a mean intercept of -0.0270 (S.D. = 0.0536, $n = 6$). ITZ had a mean slope of 0.0020 (S.D. = 0.0001, $n = 6$), and a mean intercept of -0.0341 (S.D. = 0.0299, $n = 6$). Linear regression analysis of OH-ITZ/SAP peak area ratios vs. spiked OH-ITZ concentrations gave a mean correlation coefficient of 0.9993 (range = 0.9980–0.9998, $n = 6$). Similarly, linear regression analysis of ITZ/SAP peak area ratios vs. spiked ITZ concentrations gave a mean correlation coefficient of 0.9994 (range = 0.9982–0.9999, $n = 6$). Measured OH-ITZ concentrations for the QC samples were 193.0, 446.2, and 741.5 ng ml^{-1}, respectively (accuracy $\leq -7.2\%$). ITZ determinations for the QC samples were 214.6, 440.7, and 751.1 ng ml^{-1}, respectively (accuracy $\leq 3.5\%$). Intra-day and inter-day accuracy and precision of the method are presented in Tables 2 and 3. These data indicate that the method was both accurate and precise for OH-ITZ and ITZ. In Table 2, the lowest OH-ITZ and ITZ standard (25 ng ml^{-1}) had a %RSD of 10.0 and 11.7 and a percent error of 12.6 and 6.9%, respectively; this was taken as the lower imit of quantitation. The lowest limit of detection at an attenuation of 2 (AUFS = 0.01) was 10 ng ml^{-1}.

At a column temperature of 37°C and a flow rate of 1ml min^{-1}, normal operating backpressure was approximately 101 kg cm^{-2}. Over 950 injections could be made before a significant increase in backpressure (≥ 120 kg cm^{-2}) and a subsequent loss of peak symmetry was observed. As a precaution, guard columns were replaced after 300 injections.

The HPLC method described herein offers several advantages to the previously described assays. First, the primary benefit of this method is the ability to quantitate OH-ITZ. This is a distinct advantage of any HPLC method compared to a biologic assay. The majority of the previously described HPLC assays for the determination of ITZ, including previously described precipitation methods, do not address the ability to determine OH-ITZ. Secondly, this method is rapid. For an

Table 2
Intra-day accuracy and precision for the analysis of itraconazole and hydroxy-itraconazole in human serum[a]

Concentration added (ng ml^{-1})	Mean ITZ concentration determined (ng ml^{-1})[b]	Error (%)	R.S.D. (%)	Mean OH-ITZ concentration determined (ng ml^{-1})[b]	Error (%)	R.S.D. (%)
1500	1502.2 ± 42.9	0.15	2.9	1487.1 ± 44.6	−0.86	3.0
1000	1001.7 ± 24.8	0.17	2.5	998.3 ± 24.3	−0.17	2.4
500	510.7 ± 13.2	2.1	2.3	490.1 ± 11.9	−2.0	2.4
250	244.6 ± 12.0	−2.2	4.9	249.4 ± 14.2	−0.26	5.7
100	98.6 ± 7.8	−1.4	7.9	100.1 ± 5.1	0.08	5.1
50	47.6 ± 2.7	−4.8	5.7	49.8 ± 1.3	−0.32	2.7
25	26.7 ± 3.1	6.9	11.7	28.2 ± 2.8	12.6	10.0

[a] $n = 5$.
[b] ± Standard deviation.

Table 3
Inter-day accuracy and precision for the analysis of itraconazole and hydroxy-itraconazole in human serum[a]

Concentration added (ng ml^{-1})	Mean ITZ concentration determined (ng ml^{-1})[b]	Error (%)	R.S.D. (%)	Mean OH-ITZ concentration determined (ng ml^{-1})[b]	Error (%)	R.S.D. (%)
1500	1507.3 ± 9.6	0.5	0.6	1509.5 ± 8.6	0.63	0.6
1000	991.4 ± 14.8	−0.9	1.5	991.5 ± 13.7	−0.85	1.4
500	493.6 ± 13.4	−1.3	2.7	490.6 ± 16.5	−1.9	3.4
250	47.8 ± 5.2	−0.9	2.1	249.6 ± 5.6	−0.16	2.2
100	95.8 ± 3.7	−4.2	3.9	103.0 ± 3.6	3.0	3.5
50	49 ± 2.6	−1.0	5.2	47.7 ± 4.1	−4.6	8.6
25	27 ± 3.8	8.5	13.9	26.2 ± 2.6	4.7	9.9

[a] $n = 6$.
[b] ± Standard deviation.

average analytical run of 35 samples, methods which employ several solvent extractions require approximately 6.5 h of sample preparation prior to injection. Our precipitation method reduces preparation time by approximately one-half. Using this method the preparation and analysis of 35 samples (14 clinical specimens run in duplicate plus a serum blank and six calibration samples) can be completed in approximately 12 h. Furthermore, unlike previously described methods for the determination of ITZ which used only acetonitrile as a precipitating agent, our method utilized $BaOH_2$ and $ZnSO_4$. Protein precipitation with $BaOH_2$ and $ZnSO_4$ has been used effectively as a quantitative clean-up step in the HPLC analysis of amiloride, cyclobenzaprine, cyproheptadine, diflunisal, indomethacin, phenylbutazone, and sulindac [26]. Incorporating $BaOH_2$ and $ZnSO_4$ as precipitating agents produced a very fine precipitate which upon centrifugation yielded supernates whose visible clarity was greater than when acetonitrile was used alone. The precipitating efficiency of small volumes of $BaOH_2$ and $ZnSO_4$ (50 μl) not only precluded the need for larger volumes of acetonitrile to achieve a similar degree of supernate clarity, but resultant chromatograms exhibited less endogenous interference. The third advantage is the use of a column packed with a base-deactivated stationary phase. Base-deactivation typically implies that the stationary phase support consists of high purity silica with very low levels of metal impurities and is doubly end-capped. These characteristics minimize the number of activated silanol groups which, in turn, reduce the degree of peak tailing associated with the separation of basic solutes. With conventional C-18 columns, peak tailing of bases is often minimized by increasing pH or adding amine modifiers to the mobile phase. Several published ITZ HPLC methods that employed conventional reversed-phase columns either recommended maintaining mobile phase pH greater than 7.5 or incorporated diethyl- or triethylamine into an unbuffered mobile phase [14,15,17,21,24]. Our experience with these recommendations was a dramatic decrease in column life. By switching from conventional to a base-deactivated C-18 column, we nearly doubled the number of injections that could be made (from 500 to more than 950) without experiencing a loss in peak symmetry. Lastly, in addition to these benefits our method exhibits sensitivity, accuracy and precision comparable to the previously described HPLC assays.

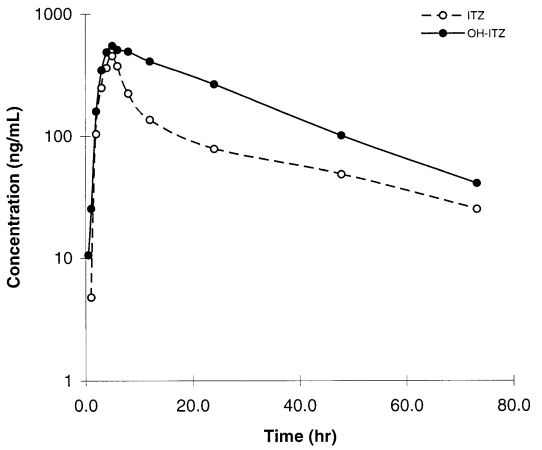

Fig. 3. Mean serum ITZ and OH-ITZ concentration–time profiles from healthy human subjects ($n = 10$) following the administration of a single 400 mg oral dose after a standard breakfast.

The described method has been used to monitor both ITZ and OH-ITZ serum concentrations in healthy volunteers, bone marrow transplant (BMT) recipients, and patients suffering from blastomycosis. A concentration–time profile of ITZ and OH-ITZ following a 400-mg oral dose of ITZ in normal volunteers is presented in Fig. 3. Because BMT recipients have particular problems of drug absorption, believed to be due to radiation or chemotherapy-induced damage to the intestinal epithelium, this HPLC method with its rapid turnaround time, has been very useful for therapeutic drug monitoring of OH-ITZ and ITZ in this population.

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